MICROORGANISMS IN SOLID MATERIALS

PHASES I, II, III, AND IV

(Including Mod. 2)

VOLUME II

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INTRODUCTION AND SUMMARY

In order to provide a high degree of reliability of microbial decontamination, it is essential that methods for the detection of viable microorganisms be developed and that these be as sensitive and reliable as possible. The detection of microorganisms within the interstices of solid materials represents a complex problem which may be approached conceptually from a number of directions. The detection methods which were studied and are reported in this document include: culturing, electron spin resonance, nonfluorescent staining, fluorescent staining, electrophoreses, and autoradiography.

A. Culturing

Culturing represents the most reliable method thus far devised for the detection of viable microorganisms. This method depends on the ability of the microorganism to undergo cellular division. There are, of course, deficiencies in the use of this method since physical and/or chemical trauma associated with the exposure of the microorganism from its location within the solid may result in a lack of ability to demonstrate the organisms' viability by culture methods. Thus, culture methods have long been established as useful methods for the detection of viable microorganisms and therefore will be used in Phases II, III, and IV.

B. Electron Spin Resonance

Electron spin resonance spectrometry involves the absorption of incident energy (usually in the microwave region) by unpaired electrons (free radicals) under the influence of a magnetic field. Electron spin resonance measures the magnetic moments of unpaired electrons and are somewhat analogous to the vibrational-rotational transitions in other forms of spectroscopy. Because free radicals occur in the process of metabolic

activity within cells, this technique was studied to determine its potential successfulness as an independent means of assessing the presence of microbial contamination in solid materials. Indeed it was found that differences existed between viable and nonviable microbial cells. Unfortunately, the order of magnitude of such differences is not sufficient to differentiate between other free radicals which may occur in a variety of nonliving solid materials (both organic and inorganic) of a wide variety of types. Since free radicals cannot be distinguished from those generated by small numbers of living microorganisms and inanimate solid particulates, this method was not considered to be of sufficient usefulness to warrant further investigation.

C. Nonfluorescent Staining

The cell walls of microorganisms contain chemical functional groups which vary in the nature and distribution of electrical charges. A wide variety of microbial stains adhere strongly to the cell walls and in some cases to the cytoplasma constituents of microbial cells. The specifity and the degree of such staining is dependent upon the specific nature and distribution of the charge groups within the cell wall and the cytoplasm. In the application of this tool for the detection of microorganisms in solids, it is essential that the organism exposed from the solid be easily differentiated by microscopy from the debris. Not only is it of importance to determine the presence of microorganisms, but it is of greater usefulness to determine whether these microorganisms are viable or nonviable. Spores of B. subtilis var. niger, Clostridium sporogenes, and Ulocladium were obtained and their viability assessed by cultural methods. An aliquot of each of these microorganisms was subjected to the killing effects of dry heat, autoclaving, exposure to ethylene oxide, formaldehyde and chlorine gas. Subsequently, both the viable and nonviable organisms were stained and examined microscopically. No differences were noted of sufficient magnitude to distinguish between viable and nonviable microorganisms.

D. Fluorescent Staining

A number of fluorescent dyes bind strongly to microorganisms. When these microorganisms are examined under a microscope equipped with suitable optics, the background may be adjusted such that it appears uniformly black while the outlines of the microorganisms present a bright fluorescence. Thus, it would appear that this technique would be more useful than the nonfluorescent staining techniques since there are fewer interfering and confusing structures in the background. This method is only useful when the solid material and other debris from the pulverized solid do not possess chemically charged groups which bind the dye. In the latter case the solid as well as the microorganism would show fluorescence and the method would be useless.

E. Autoradiography

During the process of pulverizing solid materials, structural damage to the cell wall and the cytoplasm may take place. The effects of impact, drilling, crushing, and cutting may structurally alter the cell in such a manner that cell division may not be possible. Indeed in some procedures where pulverization is allowed to proceed to extreme limits the microorganisms may be fragmented. In order to demonstrate the possible viability of such microorganisms it is necessary to utilize methods which are capable of demonstrating viability which do not depend on cell division. The incorporation for radioactivity tagged weak beta emitting isotopes provide a means for determining potential cellular viability. The presence of the tagged metabolite may then be determined by the use of radioautography. The latter technique utilizes a sensitive fine grain photographic emulsion which is sensitive to weak beta emitting radiation. Weak beta rays interact with the photographic emulsion to produce opaque silver grains in a manner somewhat analogous to the effects of light or photographic emulsion.

The presence of grain patterns which are confined within the outlines of the microbial cell or fragments thereof would provide evidence of microbial contamination if adequate controls are included. When the opaque silver grain pattern develops in regions outside the cell wall area, it is possibly

due to absorption of the radioactive metabolite by the solid or inadequate washing. The resolution of this method is highest with the weakest forms of radiation capable of activating the silver particles in the emulsion. Also resolution is dependent upon the distance between the microorganism containing the radioactive metabolite and the photographic emulsion.

Spores of B. subtilis var. niger were incubated with solutions containing tritium labeled thymidine. In this experiment it was found that as many as three weeks of exposure of the microorganism to the tritium labeled thymidine were required to provide weakly positive results. The latter experiment was conducted on glass slides so that emulsion-organism distance was optimal. It is apparent that if this experiment had been conducted on a pulverized solid previously inoculated with microorganisms, the loss of resolution would likely have been sufficient to provide no reliable evidence of microbial labeling. Therefore, this method is not suited for the assay of viable or potentially viable microorganisms in solid materials.

Thus, it appears that of the methods discussed, culturing represents the most useful and accurate method for the assessment of viability. Under certain conditions fluorescent staining may provide evidence of the presence of both living and dead microorganisms and may have limited application for determining microbial contamination. None of the other methods show promise for potential usefulness.

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PHASE I. Studies on Various Methods for the Detection of Viable Microorganisms Inoculated into Solid Materials

The objective of Phase I is to determine the effectiveness of a variety of methods for detecting viable cells. The following methods were explored in order to determine their possible usefulness as selective methods for detection of living microorganisms: culturing, electrophoresis, staining, autoradiography, and electron spin resonance. Since in the detection of viable organisms each method which could be applied might have specific advantages and disadvantages, it would be useful to have several independent methods for detecting viability. The detection of organisms by culturing is the most frequently used method of those listed. However, a devitalized cell may not grow under a specific set of cultural conditions and yet under other more ideal conditions may be capable of such growth. Thus, detection of viability by some other methods would be extremely useful. The potentially useful methods other than culture that were investigated in this phase include electrophoresis, autoradiography, and electron spin resonance.

I. PHASE I. STUDIES ON VARIOUS METHODS FOR THE DETECTION OF VIABLE MICROORGANISMS INOCULATED INTO SOLID MATERIALS

A. CULTURING

1. Introduction

a. Recovery from Solid Materials

The nature of solid substances imposes certain restrictions on the detection of microorganisms present within them. Microorganisms distributed on the outer surfaces are not as difficult to detect because of the availability of nutrients and lack of physical restrictions for cellular division to take place. Organisms located in the internal regions of solids must be freed to the extent that they are available for microscopic or cultural detection. The methods employed to achieve this may involve the use of one or more of the following principles: (1) dissolving in suitable solvent, and (2) pulverization.

In the case where the solid is dissolved, the theoretical likelihood of detecting contamination by microorganisms may be rather high. A major deficiency of this method is the toxicity of most plastic solvents to the microorganisms. Although it may be possible to detect the presence of such organisms, the likelihood of their demonstration in culture may be extremely low. Organic solvents are commonly used to disrupt permeability of microorganisms, and it would be anticipated that this generally takes place in the process of dissolving solids. The most useful application of dissolving techniques would seem to reside in microscopic scanning of stained residue obtained from the solubilized solid. Unfortunately, however, most solids do not redissolve following polymerization.

b. Nitrogen and Carbon Compound Requirements

Detection of viable microorganisms by virtue of their capacity to grow is one of the most useful methods for assessing sterility. The ability of the microorganism to assimilate nutrients from the culture medium, to convert

these nutrients to energy and structural materials for the cell, to maintain the cell's internal environment, and finally the ability of the cell to divide and provide viable daughter cells represent the requirements for cultural methods. Some bacteria require only the presence of certain trace minerals, carbon dioxide, and an inorganic source of nitrogen. Others require only a single simple form of carbon compound and utilize nitrogen from the atmosphere in place of more complex nitrogen compounds. On the opposite end of this spectrum are microorganisms which require vitamins, all seventeen natural amino acids and even traces of as yet undefined growth promotion substances. Most microorganisms lie somewhere between the two extremes.

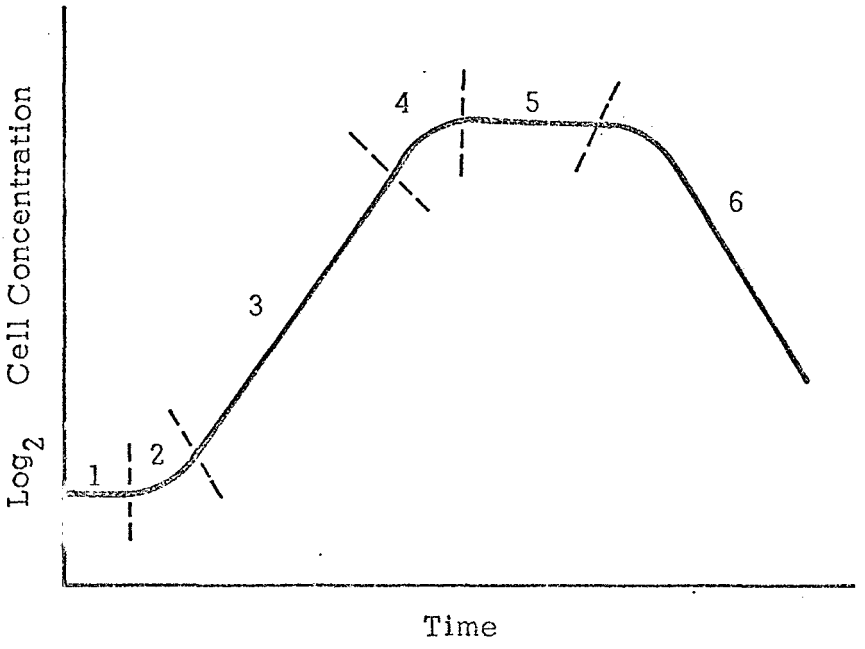
c. Growth Phases

Microorganisms pass through six distinct phases in their cultural life cycle. In the first phase cell division does not occur; however, the organisms may increase in size and accumulate metabolites. This condition exists in the lag phase (Figure I-1). A period of increasing growth rate takes place in the second or "acceleration" phase. The organisms during this phase of growth are dividing at a more rapid rate than during any of the other phases. The logarithmic phase is characterized by a constant or exponential growth rate which is followed by the retardation phase in which the growth rate is decreasing. The stationary phase is characterized by a steady state situation in which the rates of cell division and cell death are approximately equivalent. Finally, there is a phase of decline in which the number of cells dying exceed these dividing.

d. Concentration of Nutrients, Ionic Strength and Salt Concentration

The effects of concentration of nutrients on the rate and extent of growth are depicted in Figure I-2. Ideally in the absence of toxicity growth media should contain such large excesses of growth factors and metabolites that the extent of growth is not limited by their concentration.

Microorganisms are also capable of surviving under extremes of physical and chemical environments. Halophilic organisms, for example, are



Section of Curve	Phase	Growth Rate
1	Lag	Zero
2	Acceleration	Increasing
3	Logarithmic	Constant
4	Retardation	Decreasing
5	Stationary	Zero
6	Decline	Negative (more cells dying than reproducing)

Figure I-1. Growth Phases and Cell Concentration

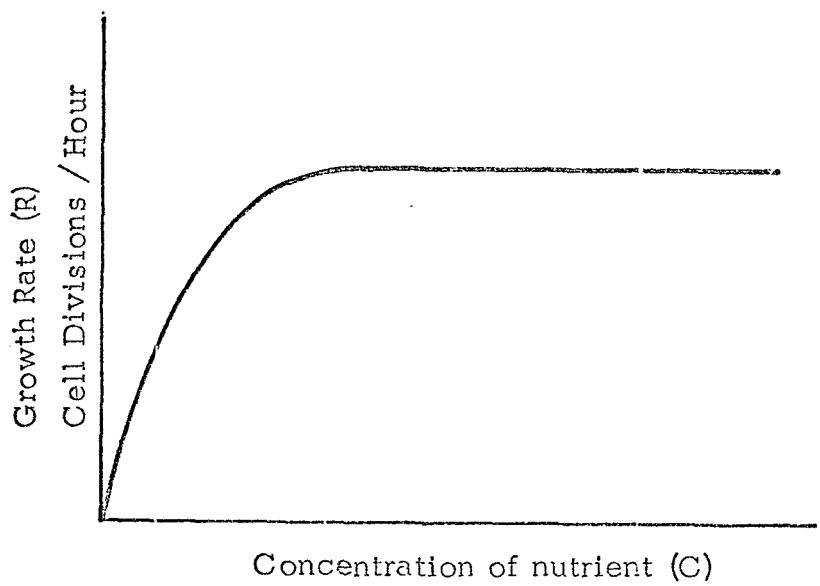


Figure I-2. Concentration of Nutrients

capable of growing in concentrated salt solutions. Some of these organisms have an absolute requirement for high salt concentration for growth. This environment generally decreases or inhibits the rate of growth of most other microorganisms. A wide variety of bacteria, on the other hand, do not grow well in the presence of even moderately low concentrations of salt. These organisms by virtue of their decreased tolerance to ionic strength may require extremely dilute growth media. The vast majority of microorganisms are able to grow at ionic strengths somewhere intermediate to these extremes.

e. pH and Physiological State

An equally important consideration is the pH of the growth media. Certain specialized bacteria are capable of withstanding the effects of and grow in the presence of strongly acid solutions. Organisms which oxidize sulfur to sulfuric acid are capable of growing in an environment which is strongly inhibitory to nearly all other microorganisms. At the opposite end of this spectrum are bacteria and fungi which accumulate basic substances in the course of growth or metabolism. Intermediate between these extremes are organisms which grow optimally at pH's in the vicinity of neutrality. Many of these organisms when growing anaerobically accumulate lactic acid as a terminal metabolic product. This accumulation causes a rapid progressive decrease in the growth rate. Likewise, organisms undergoing alkaline fermentation may eventually attain such a high pH that it interferes with growth. In these instances the presence of suitable buffers would resist such pH changes.

The pH of culture media is important as certain organisms grow only within certain limits of acidity and alkalinity. Most organisms seem to grow best at or around the neutral point, pH 7.0, but there are those which require a very acid pH. For example, certain fungi prefer pH 4.0-5.0, and Thiobacillus thioxidans is active at pH 1.0. Brevibacterium ammonia, responsible for napkin rash in babies, grows best at pH 9.0.

Sometimes advantage is taken of the ability of an organism to grow at an unusually high or low pH in order to isolate it. The media used in

recovering the Cholera vibrio from stools are made at pH 9.0, which permits the growth of the vibrio but inhibits that of most of the other organisms in feces.

The accumulation of toxic products results in a physiological condition in which the organism remains viable, but reproduces only after a prolonged lag period. After such organisms have been allowed to remain in a resting state with very small quantities of nutrients for prolonged periods of time a similar state of disability exists. This condition in either case is called the "staleing phenomenon." Such organisms are often more resistant to the effects of killing agents than organisms from the logarithmic phase of growth (Figure I-3).

f. Oxygen Tension

Some microorganisms are poisoned by the presence of oxygen in their environment. These organisms are called obligate anaerobes and, for the most part, are spore formers. In the spore stage the organism is highly resistant to the effects of dessication, oxygen tension, temperature, pH extremes, physical and chemical trauma. Conditions favorable for the proliferation of the vegetative form of these organisms, require that the oxygen be extremely low. A wide range of organisms are capable of growing under strictly anaerobic conditions even though these conditions may not be optimal. Such organisms which can grow in the presence of oxygen as well are called facultative anaerobes. At the far end of the spectrum are obligate aerobes which grow well only under high oxygen tension. These organisms are found in abundance in nearly all aerosol and soil environments.

g. Temperatures

Microorganisms grow optimally in temperature ranges between 20 and 40 degrees centrigrade. Those organisms which proliferate in soil often show a growth optimum in the vicinity of 20 to 25 degrees centigrade. whereas those found in the environment of mammals show optima between 35 and 40 degrees centigrade. Certain organisms, however, grow optimally at temperatures higher than 40 degrees centigrade. These are thermophiles.

A3

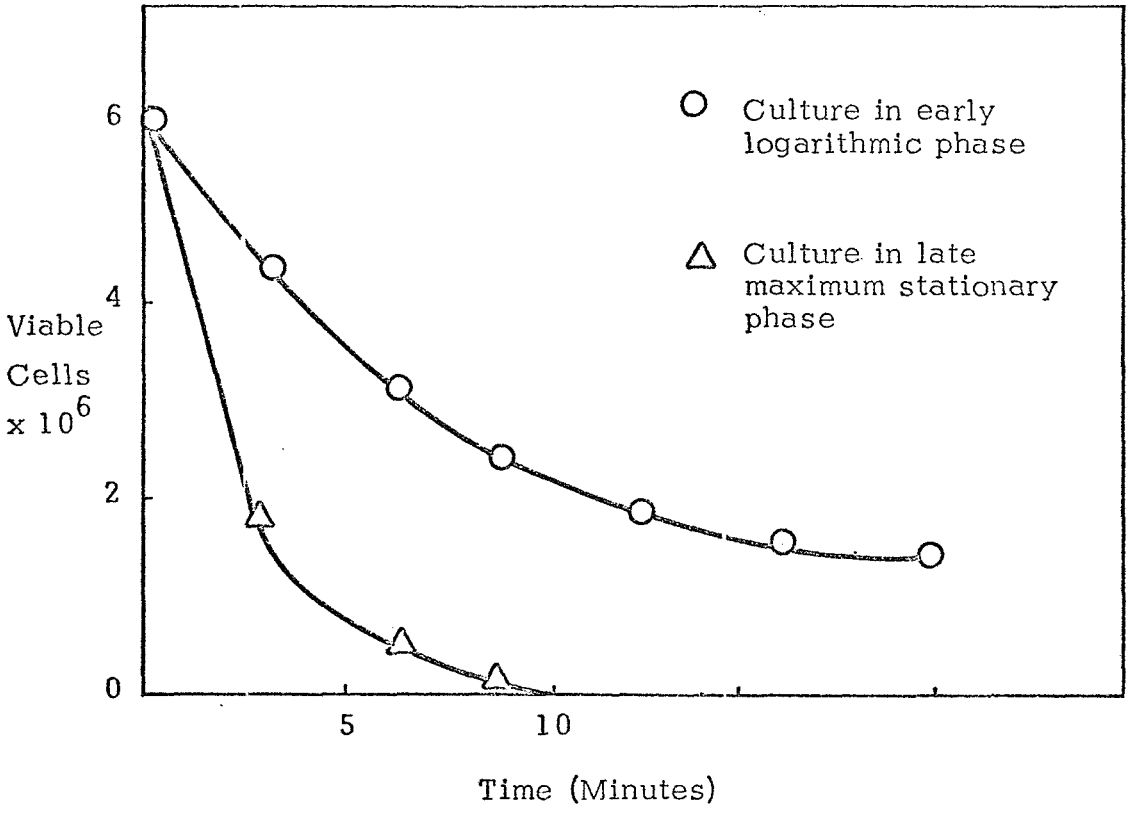


Figure I-3. Comparison between cells of young and old populations showing susceptibility to a lethal agent. Data graphed from raw data of Forbisher, 1962.

Not only can they grow at higher temperatures in the vicinity of hot springs and geysers, but they also show resistance to the effects of much higher temperature ranges.

Virtually all microorganisms can withstand low temperatures and even freezing for varying periods of time. Even though some organisms may be destroyed by the formation of ice crystals within their cell walls or by shearing effects of ice crystals as they are forming in the media, a significant percentage of all strains of microorganisms can be recovered following exposure to such temperatures.

Nearly all microorganisms can proliferate in liquid medium, although some grow well in the presence of very small quantities of moisture. The presence of moisture is particularly important when considering temperature effects on microorganisms. Resistance of spores to heat in high moisture environment is not nearly as great as that observed in the dried state. A well known protein, ova albumin, may be heated to 170 degrees centigrade in the dried state without becoming denatured. However, in the presence of water this protein is rapidly coagulated at 75 degrees centigrade. A second factor in heat resistance is manifested by the presence of colloidal substances. It is well known, for example, that the temperature required to kill lactic acid bacteria is markedly increased in cream or milk, as contrasted to water. It may be noted that the volume of most bacterial spores is roughly equivalent to one-tenth that of the vegetative form. This suggests that one reason for heat resistance exerted by the spore may be due to the concentration of the vegetative cytoplasm into a volume one-tenth that of its original volume, and hence a bio-colloid heat stability may be part of the explanation. Increased concentrations of calcium ion in the medium likewise leads to added heat stability. It has also been shown that fatty acids added to the growth medium help to impart increased resistance to heat.

2. Discussion

a. Limitations

The major limitation of culturing methods for determining sterility of

solids is that it measures only the ability of the organism to reproduce and does not measure its ability to metabolize independently of reproduction. It is conceivable that microorganisms can persist in solid materials for prolonged periods of time and yet fail to be detected by cultural methods because of deficiencies in the composition of the growth media, the procedures used to detect growth, the improper temperature or pH range, the presence of growth inhibitors which may be associated with the solid, or nonoptimal pulverization.

b. Requirements of Growth Media

Most commercially available culture media contain partially hydrolyzed proteins, peptones, and/or purified amino acids. Likewise, most of these media contain adequate quantities of vitamins. The growth media for any particular organism, however, may not be optimal in its concentration for any single constituent. It is possible for the media to be adequate in all constituents for most microorganisms and yet be marginal or deficient for a particular organism. Similarly, the growth of some organisms may be strongly inhibited by the presence of large quantities of certain media constituents.

Organisms which have been subjected to physical or chemical injury may lack the capacity for growing in an adequate medium due to cellular damage. This damage may be reversible in the case of exposure to a variety of chemical disinfectants (hexachlorophene and quaternary ammonia compounds). The latter two agents can induce sufficient damage to prevent the growth of a wide variety of microorganisms. The effects of this damage may be reversed by adding polyoxyethylene sorbitan mono-oleate and purified soybean lecithin. It is believed that these substances are assimilated by the injured organisms and that they reconstitute the damaged hydrophilic-hydrophobic cell membrane interface. Likewise, it is known that certain substances may be added to the growth media of microorganisms inhibited by ionizing radiation and that subsequent growth takes place. Little work has been done on attempts to reverse the effects of heat on microorganisms.

Although there are good theoretical grounds to support the view, at least some heat damage may be reversible.

c. Procedures Used to Detect Growth

The most common method of observing the growth of microorganisms is dependent upon visual perception of turbidity, pellicles, colonies, or floating particles. Usually such materials are stained and examined microscopically. This means of detecting growth is inadequate when such growth is slow or abortive. Detection precision can be increased by centrifuging fluid cultures which otherwise do not show growth, and examining microscopically the stained sediment.

B. ELECTRON SPIN RESONANCE

1. Introduction

Electron spin resonance spectrometry represents an application of a physical method for the study of biochemical phenomenon. Electron spin resonance involves the absorption of incident energy (usually in the microwave region) by unpaired electrons under the influence of a magnetic field.

a. Theory of Method

Magnetic resonance is the phenomenon of inducing transitions between unpaired electrons of differing energy levels. These energy transitions are analogous to vibrational-rotational transitions in other forms of spectroscopy. Electron spin resonance is concerned with the magnetic moments of unpaired electrons. Electrons spin like tops and being electrically charged create a magnetic field. The energy generated by this magnetism can be detected and measured in a magnetic field. If this spinning activated electron is also subjected to another magnetic field which is at right angles to the main magnetic field that is created, but which is pulsating, then the frequency of the pulse becomes the same as that of the frequency of precession. Then interaction will occur which will change the electron's orientation. When this change occurs, energy is absorbed. This absorbed

energy is detected in an electron spin resonance spectrometer.

b. Existence of Free Radicals in Living Systems

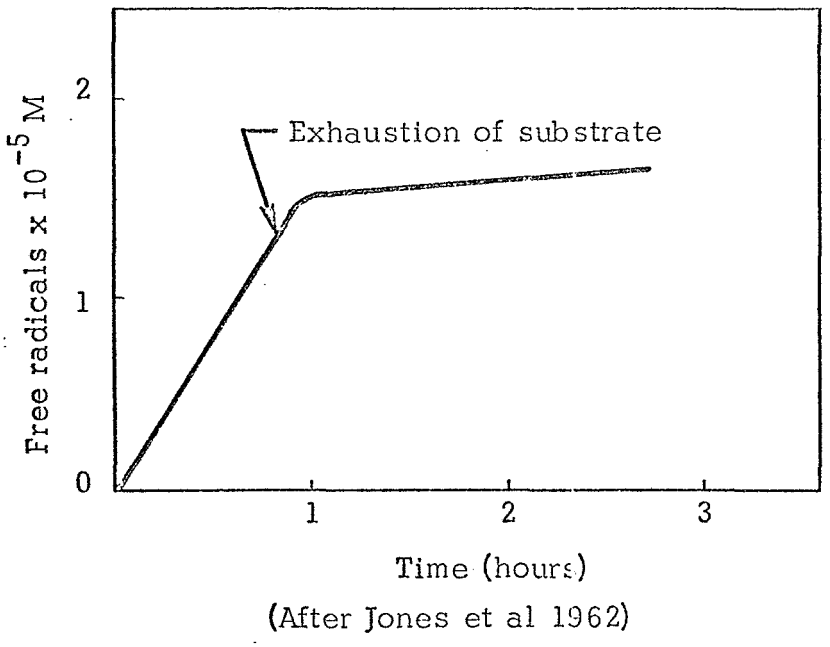
The first evidence that unpaired electrons associated with free radicals of metabolic origin were reported in freeze-dried samples of microorganisms, plant, and animal tissues. Because the free radical concentration of most microbial systems is very low (on the order of 10^{-7} to 10^{-5} M) extremely sensitive instrumentation is required. The volume of most electron spin resonance detection containers is on the order of 0.1 to 0.3 cm^3. Electron spin resonance studies of oxidation reduction enzyme systems provide a possible means of detecting enzymes. Michaelis in 1940 suggested that although many enzymatic oxidation reduction processes appear to involve two electron transfers, electrons are actually transformed one at a time after the first step.

Carefully detailed electron spin resonance investigations of succinc acid dehydrogenase have been particularly useful in providing information regarding the role of free radicals in enzyme catalyzed processes (Figure I-4).

Major sources of unpaired electrons in living systems include:

1) Respiratory enzymes
   a) flavins (flavin mono nucleolide, flavin adenine di nucleotides)
   b) cytochromes b, b_1, C, C_1, a, a^3
   c) hemoglobin, myoglobin
2) Metalloflavo proteins (succinic acid dehydrogenase, anthine oxidase, aldehyde oxidase, nitrate reductase, sulfate reductase, aldehyde reductase)
3) Enzymes concerned with photosynthesis

In addition, an excited electron may travel through a continum of fibrous proteins. It has been suggested that such proteins may link the insoluble outer fibrous proteins with the insoluble oxidation enzymes of the cell. Desoxyribonucleric acids (DNA) also may contain excited electrons



(After Jones et al 1962)

Figure I-4. Free Radicals Formed by the Acid by Succinic Dehydrogenase

following excitation. Biological systems carrying out either oxiditive or reductive pathways would thus create a steady state stream of unpaired electrons. As would be expected when the living organism is no longer in the living state where unpaired electrons would be found. Unpaired electrons appear to be essential components of living organisms by virtue of their necessary role in biological oxidation and reduction mechanisms.

Other types of systems exist in which paramagnetic electrons may be demonstrated. Wool represents a material produced by a living system but which is a nonliving tissue and following electrical or light excitation, contains unpaired electrons. Szent-Györgyi in 1946 described the presence of impaired electrons in gelatin dyed with various ionic dyes. The paramagnetic spectrum of hemoglobin is shown in Figure I-5. The spectrum of the amino acid glutathione is shown in Figure I-6.

2. Methods

In an exploratory test to determine whether the effects noted by Heckly and Dimmick might occur in other types of cells, the following steps were taken. Spores of B. subtilis were placed into each of several quartz tubes. These tubes were 9 inches long and 2 mm in diameter in order to fit the sample holder in the Varian EPR spectrometer. The tubes were sealed on one end but open to the atmosphere on the other. Except for those in one tube, the spores had been dried from suspension in acetone and were in the form of dry, unground, lumpy powder. They numbered 2.5×10^{11} spores per gram of dry powder and each tube contained about 0.1 gram of them. From the original container, the spores were sifted into a sterile beaker suing a sterile fine-mesh screen sieve. By means of a small glass funnel they were then transferred into the quartz tubes. Yeast, in the form of dry active cells (Fleishmanns baking yeast) (approx. 5×10^9 cells) were similarly dispensed into quartz tubes.

Using a disposable glass syringe, 0.1 ml of the liquid phase of each of the biocides; ethylene oxide, ethylene imine, acrolein, acetone, or peracetic acid (40%) was placed on top of the organisms in the tube.

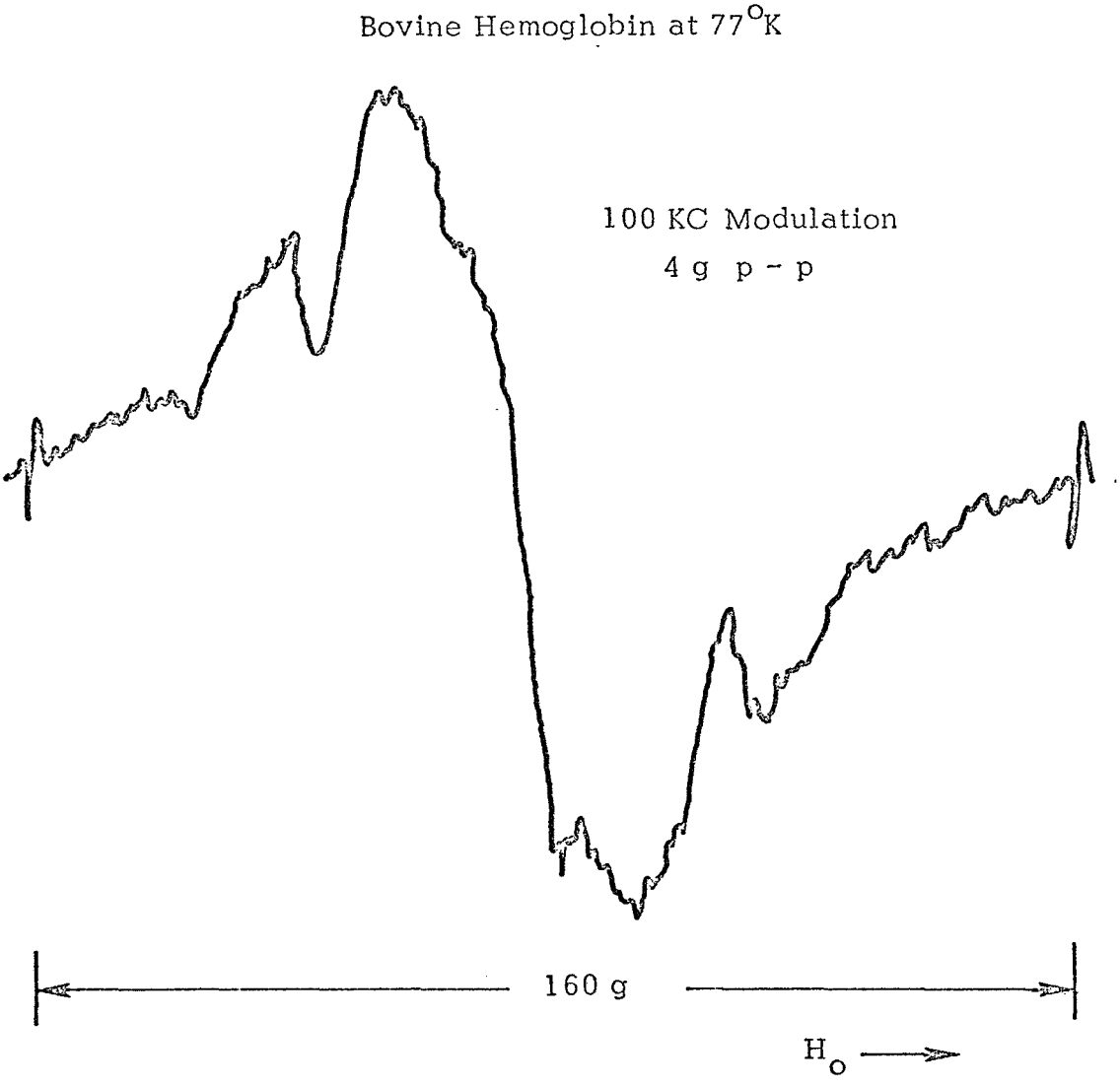


Figure I-5. First derivative trace of paramagnetic resonance absorption developed in bovine hemoglobin after ultraviolet irradiation at 77°K. Only the weak pre-irradiation absorption could be detected in this sample when it was warmed to room temperature. H_1 field was approximately 0.07 gauss.

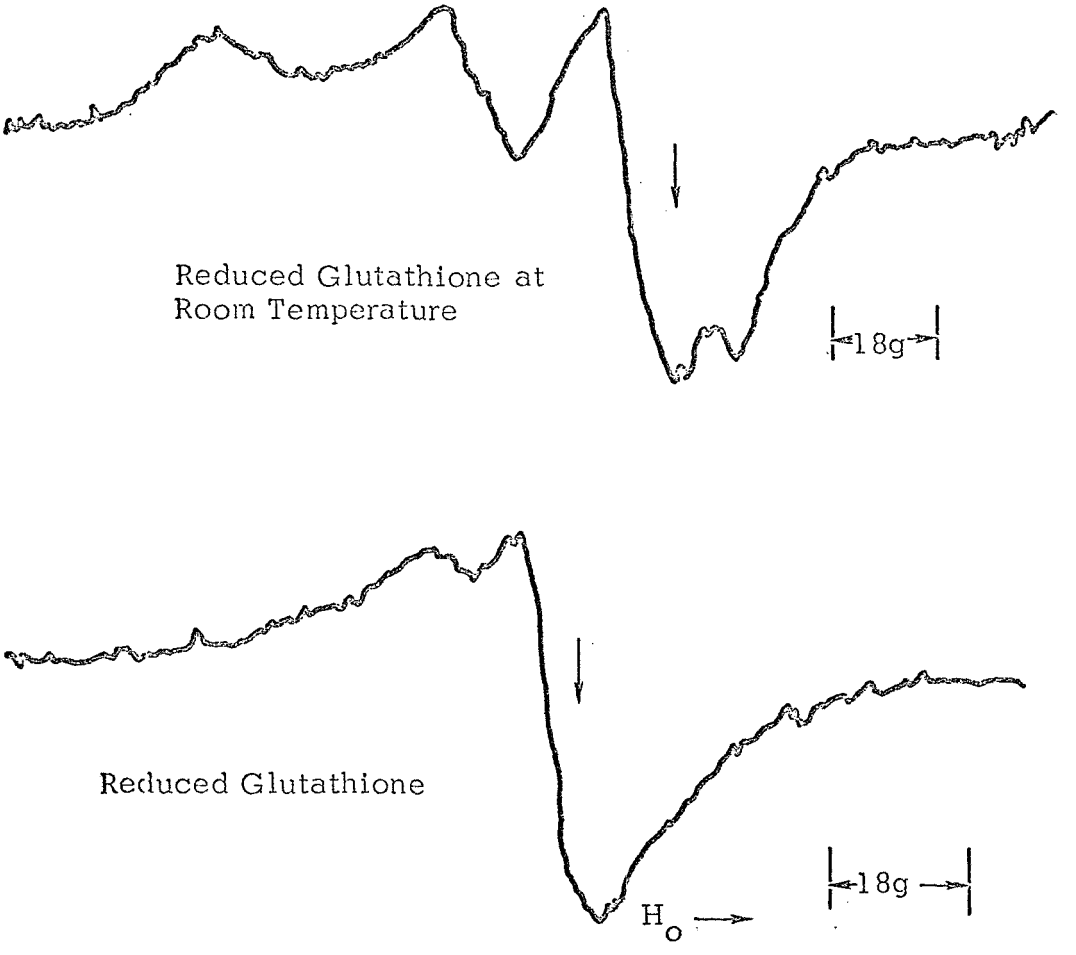


Figure I-6. First derivative traces of paramagnetic resonance absorption in reduced glutathione. This compound is a tripeptide composed of glutamic acid, glycine and cysteine. This compound, in the form of a dry powder, was placed in a tube and the tube was evacuated in an attempt to remove oxygen. The tube was then sealed and the compound was cooled to 77°K and irradiated with ultraviolet light. During the absorption measurements at 77°K, the 100 KC modulation amplitude was 0.6 gauss peak-to-peak and H_1 approximately 0.15 gauss at room temperature, the 100 KC modulatior was 2.0 gauss peak-to-peak, the H_1 field strength was unchanged.

3. Results

The bacterial spores with and without ethylene oxide, chlorine, or ethylene imine gave a very strong signal (in excess of 1 millivolt). The yeast gave a weak signal which was the same whether or not they had been treated with biocide. Heating the tubes gently produced a darkening of the contents and an increase in signal. This same effect of heating (charring) occurs with sugar, however.

4. Discussion

A vast variety of materials derived from nonliving sources are also known to have unpaired electrons. Riehl (1955, 1956) has considered the energies, ϵ_1, to transfer an electron from a molecule in a crystal lattice to its nearest neighbor in the lattice, and ϵ_2 to transfer an electron from a molecule to a distant site within a lattice. These quentited according to Lyone (1957) are:

$$\Delta \epsilon_1 = I - A\, e^2/r - P\mu$$

$$\Delta \epsilon_2 = I - A - 2Pi$$

Here I is the ionization potential, A the electron affinity of the molecule in vacuo, e/r is the Coulombic energy of the ion pair, $P\mu$ the polarization of the crystal due to the ion pair, and 2Pi the polarization of the crystal due to the separated ions. Due to the vast range of possible compound crystals and types of interactions in electronic components and component materials, it would be expected that paramagnetic signals could occur in nearly any range and could obscure signals found from living cells (due largely to paramagnetic species of carbon oxygen, sulfur, nitrogen, copper, iron, molybdenum and zinc).

5. Conclusions

Clearly, ESR, if it were effective in detecting the presence of living organisms in solid materials, would offer the possible advantage of non-destructive testing. Unfortunately, however, it is not possible to reliably

distinguish between living spores and dead spores, living yeasts and dead yeasts, or even charred material from living material. The difference in EPR signal emplitude in rapidly metabolizing, slowly metabolizing, and dead cells is not sufficiently large to provide data which would be useful even as a "screening" method.

Many solids derived from purely nonliving matter provide strong EPR signals. Examples of these materials are:

diphenyl picrylhydrazine
phthalocyanine
anthracene
dimethyl aniline-bromanil complex

In addition to the compounds on this list are many others including plastics mixed with metals.

EPR spectroscopy would not be useful as a method for the detection of living (or even nonliving) microorganisms in solid materials.

C. STAINING

1. Introduction

a. Theory of Methods

A wide range of dyes are available whose staining characteristics on microorganisms have been investigated. The binding affinity of the stain for the microorganism may reflect discrete and sometimes relatively specific chemical binding sites on the cell wall or cytoplasm of the organism. For example, Alican Blue is a dye which binds to acidic substances and is useful for showing the presence of acid mucopolysaccharides. A wide range of other dyes characterized by relatively strong and nonspecific staining properties are well known. The latter category includes most bacteriological stains, examples of which are methylene blue, Azure II, Gentian violet, and most of the triphenyl methane dyes. During the course of staining by these dyes, microorganisms are usually rendered nonviable.

As a rule, those organisms which are not stained by a particular dye may have cell walls which are protected by fatty or waxy coats, or the electrical charges on the dye molecule may be similar to that of the cell wall of the unstained organism and electrostatic repulsion prevents the dye from binding to it.

b. Potential Use with Solid Materials

When bacteriological stains are interacting with microorganisms, the binding affinities of the dyes for the organisms become highly critical. If the affinity of the dye for the microorganism is higher than that of the solid fragments of the materials, contrast will be apparent. This contrast is highly important and should under idealized conditions result in an unstained background of solid fragments and intense, characteristic staining of the microorganism. Theoretically one of the methods which could be applied to provide such staining characteristics involve the incubation of the pulverized solid with very dilute stain solution. When the microorganism binds the stain and the background of solid material fragments do not stain, the presence of microorganisms may be established. A second type of approach may be used in which the pulverized solid is treated with a more concentrated stain with the object of obtaining maximal staining of microorganism even though the pulverized solid fragments may stain intensely. This over-stained material may then be destained by treatment with a solvent in which the dye is very soluble. In some cases destaining may be accomplished by treatment with ether, acetone, or other nor aqueous solvents. Another approach to destaining is to use acid or alkaline solutions which reverse the binding of the dye to the surface of the solid particles, thus facilitating its removal with the solution leaving the microorganisms selectively stained.

One of the major limitations of dye staining technique is that the microorganism must possess a characteristic structure in order to differentiate it from stainable debris. Artifacts would thus obscure organisms which are distorted or which do not have distinctive shapes. Fungal elements and yeasts are usually less difficult to distinguish because of their more easily

distinguished intracellular detail and the presence of a well developed cell wall.

2. Methods

A mixed inoculum of Serratia marcescens, Bacillus subtilis, Clostridium sporogenes and Ulocladium was inoculated into solid rocket propellant and a solid epoxy plastic material (Maraset*). The inocula in both cases consisted of 10^3 of each organism/cm^3 of the propellant or plastic. The solids were pulverized by sawing with a hack saw type blade.**

Approximately 100 mg of each of the solids were stained using the following stains:

1) Gram stain
2) 2.0% methylene blue
3) Alican blue
4) Carbol fuchsin
5) Malachite green

Destaining was attempted with the following solvents:

1) Water
2) 2% HCl in water
3) Acetone-alcohol
4) Chloroform

Each of these destaining solutions were visually controlled by microscopic observation.

3. Results

The backgrounds of the stained specimens were unsatisfactory in all cases. It was not possible in most cases to clearly differentiate the

*Marblette Corp. 37-31, 30th St., Long Island City, N. Y.
**Blade No. 1218-3, Clemson Bros., Inc.

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spores from the background debris. The appearances of the stained organisms are shown in Tables I-1, -2, -3. The background consistently was nonuniform in appearance. The conidia (spores of fungi) could be seen and differentiated in all stained preparations; however, scanning was tedious and many stained fragments could not be unequivocally distinguished from debris.

The propellant was opaque and stained intensely. Destaining did not lead to improvement of microscopic detection of microorganisms. The staining methods as applied in this study were not useful in the detection of microorganisms in solid propellant.

4. Discussion

Staining methods are useful for detecting microorganisms under certain specified conditions. If the background of the microscopic field is sufficiently uniform they may be easily differentiated from the debris. In the process of pulverizing solid materials, fragments of various sizes and shapes and possessing varying optical properties generally obscure the outlines of microorganisms and increase incidence of artifactitious interpretation is possible.

No differences were observed between stained viable and nonviable spores of B. subtilis.

5. Conclusions

Staining of pulverized solids using a variety of bacteriological stains was relatively ineffective for demonstrating the presence of bacteriological spores. Less difficulty was encountered in some instances in the Maraset plastic of detecting fungal hyphal and conidial structures than with the bacterial spores. Detection of microorganisms by staining of propellant was more difficult than with stained Maraset plastic.

Staining with the stains used in this study did not result in detectable differences between viable and nonviable organisms.

TABLE I-1

VIABLE AND NONVIABLE

Staining Characteristics of Spores of B. subtilis, var. niger

Stain	Spore Treatment	Color of Spores	Color of Vegetative Cells	Dispersion
Gram stain	Viable Untreated	Pink outlined in purple	Cells at periphery are pink	Well dispersed
	Heated dry	Blue to dark blue	Dark red	Clumped
	Autoclaved	Intense blue	Pink to red	Well dispersed
	Ethylene oxide treated	Same as for viable spores	Same as for viable spores	Well dispersed
	Chlorine treated	Intense dark green	Dark red	Clumps

TABLE I-2

VIABLE AND NONVIABLE

Staining Characteristics of Spores of B. subtilis, var. niger

Stain	Spore Treatment	Color of Spores	Color of Vegetative Cells	Dispersion
Malachite green spore stain	Viable Untreated	Emerald green	None	Spores central well dispersed
	Heated dry	Intense dark green, black center	None	Clumped spores
	Autoclaved	Intense green	None	Well dispersed. Similar to viable cells.
	Ethylene oxide treated	Emerald green	None	Well dispersed

TABLE I-3

VIABLE AND NONVIABLE

Staining Characteristics of Spores of *B. subtilis*, var. *niger*

Stain	Spore Treatment	Color of Spores	Color of Vegetative Cells	Dispersion
Carbol fuchsin spore stain	Viable Untreated	Red	Light pink	Well dispersed
	Heated dry	Intense dark red	None	Clumped
	Autoclaved	Intense dark red	Light pink	Well dispersed
	Ethylene oxide treated	Same as for viable spores	Same as for viable spores	Well dispersed
	Chlorine treated	Intense dark red	None	--

D. FLUORESCENT STAINING

1. Introduction

a. Theory of Method

Using light microscopy and nonfluorescent dyes the background of the microscopic field is bright and contains the detailed and confusing outlines of opaque or semi-opaque solid fragments which may or may not stain the same color as the organism.

Microorganisms may be detected within solids more readily by the use of fluorescent staining. Organisms stained with a fluorescent dye may be visualized microscopically using an ultraviolet light source. The number of fluorescent dyes which have proven satisfactory for the staining of microorganisms are much smaller than those nonfluorescent dyes used for routine bacteriological staining purposes. However, there are distinct advantages to the use of ultraviolet fluorescent microscopy. The fluorescent light source system may be adjusted to provide a microscopic background field which is essentially black. The particles of solids which do not take up the fluorescent stain therefore would not be visualized. Ideally, the background should be as nearly black and uniform as possible. The organism which has taken up the fluorescent dye would appear as a bright fluorescent structure against a contrasting dark or black field. This, of course, facilitates the task of detecting small numbers of organisms in a pulverized sample.

Ideally, in the evaluation of the contamination level of solid materials, staining procedures should be able to detect relatively small numbers of viable cells, preferably as few as one. Such requirements indicate that the ultraviolet fluorescent technique should be best suited to the job. For this reason, most of the literature survey and the subsequent experimental work emphasizes ultraviolet fluorescent techniques.

b. Differentiation Between Living and Dead Cells

Very few reports were found in the literature in which the use of stains or dyes to distinguish viable from nonviable microorganisms was

described. Only one was found that was directly related to viability of microorganisms (Meisel 1961). Meisel reported that the fluorescent dye, primuline, could be used to distinguish between living and dead microorganisms. The report of the experimental procedures was somewhat brief but it did state that dead cells fluoresced much more intensely than did live ones. This effect was demonstrated by using live organisms in the dye on a microscope slide and heating the slide while it was under observation. As the heat killed the cells they showed intense fluorescence. The effects were reported for vegetative cells.

Several investigators have reported that differentiation between living and dead cells, other than microorganisms, was possible with the fluorescent dye, acridine-orange. Yurtsev (1960) used the dye to determine pollen viability in certain cereals. Vinegar (1956) reported differential staining of living and dead ascites tumor cells with acridine-orange. This dye has been used to distinguish between cancerous and noncancerous cells and is the basis for rapid screening techniques on cervical and vaginal smears. Van Niekerk (1962) reported a procedure using acridine-orange to detect pathogenic vaginal flora in vaginal smears. Both bacteria and protozoa were stained by the dye. There are several descriptions (Riva, 1962, Anon., Stain Tech., 1962) of the use of acridine-orange for staining of tissue sections and for exfoliative cytology studies. While there were some minor modifications in all the reported procedures using acridine-orange, all used dye dilutions of from 1:5,000 to 1:10,000 in an acid (pH 3 to 5) buffer.

Oginsky and Umbreit (1955) point out that cells of certain microorganisms lose acid fastness (stain not removable by acid solution) when subjected to mechanical damage. The possibility is suggested that loss in acid fastness might also be a consequence of loss in viability. One of the staining procedures for bacterial spores is based on an acid fast technique using carbol fuchsin (not a fluorescent dye). Auramine-O is a fluorescent dye used to stain acid fast tubercle bacilli and might be useful in distinguishing between viable and nonviable spores. These spore-staining and acid-fast staining techniques are a portion of standard laboratory procedure (Conn 1957).

Another potentially useful fluorescent material is the antibiotic, tetracycline. While not considered to be a biological dye, this antibiotic is absorbed by many types of cells and does fluoresce in ultraviolet light. This material has been used to identify growing bone (Hattner 1962) debris in the region of cancerous growths (Milch 1961; Vassar 1962), certain parasites (Tobie 1960), and the mitochondria of cells (DuBuy 1961) by the fluorescent technique. One theory of the action of the tetracyclines is based upon the binding of calcium ion (Anon., Chem. Eng. News, 1962), and action upon enzymes which require calcium similar to that of some chelating agents. Riemann (1961) has shown that germination of spores can be greatly stimulated by the use of chelating agents in the presence of the proper calcium concentration. It is possible that in the presence of calcium, tetracycline could act as a spore germination stimulant with the result that viable spores would have a greater uptake of tetracycline than nonviable ones. If this were the case, a possible staining procedure for distinguishing viable from nonviable spores might result.

Other fluorescent biological dyes are available (Anon., Reichert, 1963) for staining microorganisms. The fluorescence and absorption spectra for many of these dyes are available (Porro 1963).

c. Fluorescent Antibody Technique

One of the fluorescence techniques which has been the subject of extensive investigation since its introduction in 1941, is that of the fluorescent antibody technique. In one form of this technique, an antibody is prepared, in some appropriate laboratory animal, against a specific microorganism. The antibody is labelled with a fluorescent molecule. When the labelled antibody comes in contact with the antigen (in this case, some portion of the specific microorganism) the antigen-antibody reaction site is marked with the fluorescent label. This technique, as well as several modifications, has been used successfully in the rapid and quite specific identification of microorganisms. It is sufficiently sensitive that a single microorganism can be detected.

2. Methods

a. Fluorescent Dyes

The four fluorescent dyes selected for investigation were tetracycline (or one of its derivatives), primuline, acridine-orange, and auramine-O. No staining procedures using tetracyclines were found in the literature so that preliminary studies to develop an effective procedure were made. Some exploratory experimentation with primuline was also necessary. Procedures for acridine-orange and auramine-O were taken directly from the literature.

b. Staining Procedures Used

(1) Tetracycline. Two tetracycline compounds were selected for the initial screening, tetracycline itself and dimethylchlorotetracycline*. The latter compound gave the stronger fluorescence and was used in subsequent procedures. A 1/3 fractional factorial design was used with the following factors being investigated at three levels: pH of the buffer used to dissolve the dye, $CaCl_2$ concentration, exposure time of the microorganisms (B. subtilis spores) to the dye-$CaCl_2$ mixture, and the pH of the buffer used to mount the coverslips on the slides. On the basis of this experiment it was found that the intensity of the fluorescence of the spores was neither dependent on the pH of the buffer used to dissolve the dye nor on the $CaCl_2$ concentration (including none). There was a small effect of exposure time (one hour being best), and a strong effect of the pH of the mounting buffer. In the latter case, the more alkaline buffers were better, pH of 9.0 being the highest used.

A second experiment was performed in which the dye concentration and the type of buffer for coverslip mounting were the primary factors. Two buffer systems were used, one aqueous and one with glycerine. The results indicated that the concentration of the dye was not a critical factor. The glycerine buffer (pH of 9.0) was considerably better, however, than an aqueous buffer of the pH.

*These materials were donated by Lederle Laboratories as Achromycin and Declomycin, respectively.

On the basis of these two experiments, the following procedure was used:

1. Prepare an 0.5% solution of Declomycin in Sorensen's phosphate buffer, pH of 7.0.
2. To one ml of this dye solution add three drops of a B. subtilis spore suspension containing approximately 10^9 spores/ml.
3. Incubate for one hour at 37°C.
4. Centrifuge, remove, and discard the supernatant.
5. Resuspend the centrifuged spores in glycerine buffer, pH 9.0. This buffer is prepared by adding 9.8 parts of glycerine to 0.2 parts phosphate buffer, pH 9.0.
6. Place one or two drops of the suspension on a microscope slide, cover with a coverslip, and examine with ultraviolet light using oil immersion objective.

(2) Primuline. Meisel (1962) gave very little information on the preparation of the dye. Primuline is soluble in alkaline solutions and moderately insoluble in acid. Two pH's, 7.0 and 9.0, were selected for study and two primuline concentrations were used, 1:10,000 (one gram in 10,000 ml buffer) and 1:100,000. Because of the relatively small number of variables, no preliminary experiments were made. The following procedure was used:

1. Prepare four solutions of primuline of 1:10,000 and 1:100,000 dilution of phosphate buffer, pH of 7.0 and 9.0.
2. To one ml of each solution add 3 drops of the spore suspensions being studied (approximately 10^9 spores/ml).
3. Incubate 30 minutes at 37°C.
4. Centrifuge, remove, and discard supernatant.
5. Resuspend spores in phosphate buffer, pH of 4.4.

6. Place one or two drops of suspension on microscope slide, cover with coverslip, and examine.

(3) Acridine-Orange. The procedure of Van Niekerk (1962) was used and is as follows:

1. Prepare smears of spore suspensions on microscope slides and allow to air dry.
2. Fix smear in 95% ethyl alcohol and diethyl ether (1.1) for 10 minutes.
3. Pass slide quickly through rinses of 80, 70, and 50% ethyl alcohol.
4. Leave in distilled water for 2 minutes.
5. Put in citric acid-disodium phosphate buffer, pH of 3.8, for 3 minutes.
6. Stain in 0.01% acridine-orange in pH 3.8 buffer for 3 minutes.
7. Put in clean buffer, pH of 3.8 for 4 minutes.
8. Mount coverslip with same buffer.
9. Examine.

(4) Auramine-O. The procedure of Richardson and Miller (Conn 1957) was used and is as follows:

1. Prepare solution "A"

Auramine-O (94% dye content)	0.1 gm
Liquified phenol	3 ml
Distilled water	97 ml

2. Prepare solution "B" (must be freshly prepared)

Ethyl alcohol (70%)	100 ml
Conc HCl	0.5 ml
NaCl	0.5 gm

3. Prepare smears and air dry.

4. Stain in solution "A" for 2-3 minutes.

5. Wash in tap water.

6. Destain in solution "B" for 3-5 minutes.

7. Dry slide and examine with high dry objective.

(auramine-O does not fluoresce in solution).

c. Spore Suspensions

Viable spore suspensions were prepared by dry B. subtilis spores. Nonviable spore suspensions were prepared by exposing dry viable spores to ethylene oxide, autoclaving, dry heat, and chlorine gas. Viability and nonviability were verified by culturing on Trypticase soy agar plates. Final concentrations used for the staining procedures was approximately 10^9 spores/ml.

d. Microscopy

Ultraviolet fluorescent microscopy was performed using an American Optical binocular microscope equipped with a dark-field condenser. Objectives used were either a 43X high dry or a 97X oil immersion with an integral iris diaphragm. Oculars were 10X. The light source was a Leitz Model 250 using an Osram HBO 200 high pressure mercury vapor lamp. Filters available for the light source were a 4 mm red suppression filter, BG-38, a 2 mm heat absorbing filter, KG-1, a 2 mm ultraviolet fluorescence filter, UG-1, and a 3 mm blue fluorescence filter, BG-12. Available barrier filters were Wratten 2A, 2B, 2C, and 15G. For all of the studies the following combination was used: 2 mm KG-1, and 2 mm UG-1 lamp filters and a Wratten 156 barrier filter.

3. Results

a. Tetracycline

The first experiment compared the fluorescence of viable and

nonviable spores. Duplicate slides were used. Fluorescence intensity was judged semiquantitatively as + through ++++.

TABLE I-4

Tetracycline Fluorescence of Viable and Nonviable Spores of B. subtilis var. niger

Spore Suspension	Replicate	Fluorescence
Viable	1 2	++++ ++++
Autoclave killed*	1 2	
Dry heat killed**	1 2	++++ ++++
Chlorine killed**	1 2	+++ +++
Ethylene oxide killed	1 2	++++ ++++

*spores failed to centrifuge
**spores markedly distorted

These results indicate no significant difference between the staining of viable and nonviable spores. In order to check the possibility that staining differences, if present, might be more apparent for short incubation times, a second experiment was performed in which incubation periods of 1, 5 and 15 minutes were used.

TABLE I-5

Effect of Incubation Time on Tetracycline Fluorescence Staining of Spores of B. subtilis var. niger

Incubation Time	Spore Suspension	Fluorescence
1 minute	Viable	++
	Autoclaved	++
	Heated dry	+++
	Chlorine treated	++
	Ethylene oxide treated	++
5 minutes	Viable	++
	Autoclaved	++
	Heated dry	+++
	Chlorine treated	++
	Ethylene oxide treated	++
15 minutes	Viable	+++
	Autoclaved	+++
	Heated dry	++++
	Chlorine treated	+++
	Ethylene oxide treated	+++

Although there appears to be slightly greater fluorescence with the dry-heat killed spores than with the others, the difference is not sufficient enough to be the basis of a realiable differentiation technique.

b. Primuline

For the primuline experiment, spore suspensions were limited to viable and dry-heat killed. Two primuline concentrations and two buffer pH's were tested.

TABLE I-6

Effect of Primuline Concentration and pH on Fluorescence Staining Intensity of Viable and Nonviable Spores of B. subtilis var. niger

Primuline Concentration	Buffer pH	Spore Suspension	Fluorescence
$1:10^4$	7.0	Viable	++
		Heated dry	+++
	9.0	Viable	++
		Heated dry	+++
$1:10^5$	7.0	Viable	++
		Heated dry	+++
	9.0	Viable	++
		Heated dry	+++

Here again, there was a slightly greater fluorescence with the heat killed spores than with the viable ones. The differences, however, were not great enough by this procedure to permit a reliable differentiation between viable and nonviable spores.

c. Acridine-Orange Fluorescent Staining

The results are as follows:

TABLE I-7

Spore Suspension	Flucrescence
Viable	+
Autoclaved	+
Heated dry	++
Chlorine treated	+
Ethylene oxide treated	+

d. Auramine-O

The results are as follows:

TABLE I-8

Auramine-O Fluorescent Staining of Viable and Nonviable Spores of B. subtilis var. niger

Spore Suspension	Fluorescence
Viable	+++
Autoclaved	+++
Heated dry	+++
Chlorine treated	+++
Ethylene oxide treated	+++

No differenced between viable and nonviable spores was found.

4. Conclusions

a. No significant, practical differential staining reactions were found for any of the four staining procedures tested.

b. There appears to be a slight increase in the fluorescent intensity of dry-heat killed spores compared to viable spores or to spores killed by other means.

c. Fluorescent intensity for the above procedures was greatest with tetracycline, intermediate with primuline and auramine-O and least (weak) with acridine-orange. All procedures stained both spores and vegetative forms (some vegetative forms were present in the spores suspensions).

d. Though no distinction between viable and nonviable contamination inside solids, through staining techniques, appears to be possible, detection of the contamination is quite possible and feasible for many solid materials. In combination with culturing, staining can increase the sensitivity of the detection technique

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when the metabolizing cells act on ingested stain (Salberg 1960).

e. In none of the fluorescence techniques nor the conventional staining techniques described in Table I-1 did the ethylene oxide killed spores appear different from the viable spores.

E. ELECTROPHORESIS

1. Introduction

a. Theory of Electrophoresis

Electrophoresis is a method of separating particles, microorganisms and macromolecules which depends upon their electrical charges and molecule shape and weight. When particles with an electric charge are placed in an electric field they will move toward the electrode possessing an opposite electrical charge, (Figure 1-7). The direction of migration is determined mainly by the charge in the particle. Particles with different charges will move with different velocities and thus can be separated from one another.

b. Potential Application for Separation of Living from Nonliving Cells

Life processes affect the electric charge-to-mass ratio of cells. The life processes going on in the living cell tend to maintain electric charges of characteristic types and magnitudes for particular cells. When populations containing both living and dead cells are suspended in a fluid in an electric field the two types may exhibit different mobilities. This technique if it were effective might be useful in determining death-rate mechanisms because in any particular sample it is almost impossible to observe all the cells, both living and dead. The ratio of cells exhibiting the two different electrophoretic mobilities would be measured.

The distance through which a particle moves under the influence of an electric current is proportional to the time and to the electrical potential gradient at the site where the particle is. The potential gradient in turn depends upon the current, the conductivity of the solution, and the cross-sectional

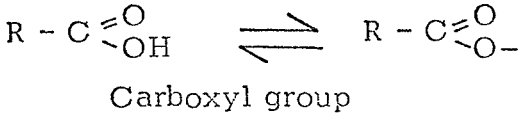
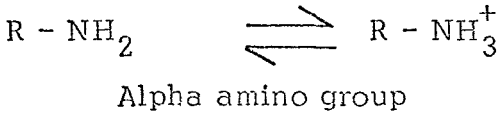
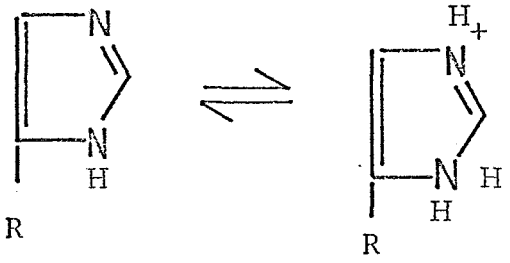
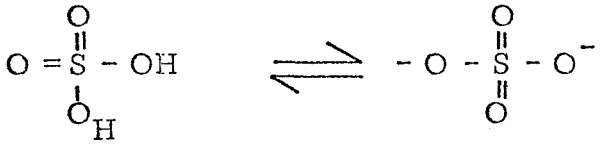
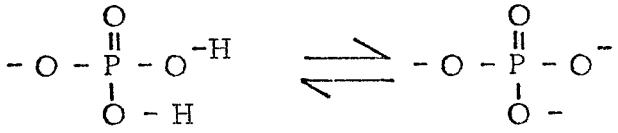
Functional groups which appear in cell membranes and cell walls	Approximate pK value (pH at which 50% of functional group is ionized)
 Carboxyl group	3.8
 Alpha amino group	9.2
	7.8
	< 2
	

Figure I-7. Theory of Electrophoresis

area of the solution at this point. The proportionality constant is known as the electrophoretic mobility.

2. Methods

a. Description of Apparatus

A standard rectangular microelectrophoresis cell was utilized in the study in which the mobility of the cells was observed by a microscope with a total magnification from 200 to 1000X. The velocities were determined by focusing on a given particle and measuring the time required for the particle to traverse a given distance using a calibrated ocular micrometer. The microscope was focused at one of two planes in the microelectrophoresis cell. These two planes are referred to as the stationary planes. In an electrophoresis cell of this type the observed velocity of the particles will be composed of two components. One component is due to the true electrophoretic forces on the particle and the other is due to a motion of the fluid itself within the electrophoretic cell (the electro-osmotic streaming effect). The latter phenomenon is due to the effect of the electrical field on the suspending fluid. For a closed cell, as is used for electrophoresis studies, the velocity due to electro-osmotic streaming is a maximum on the centerline in one direction and is in the opposite direction along the walls.

At some plane between the cell center line and the wall the streaming velocity is zero. These are the stationary planes. The motion of particles in these planes is due solely to the electrophoretic mobility and these are the planes upon which the microscope must be focused. The isoelectric point is determined by finding the pH of the suspending fluid for which the particle velocity in the stationary plane is zero. This was done by measuring the velocity for a range of pH's on either side of the isoelectric point and plotting velocity against pH. The pH where the curve crosses the zero velocity line is the isoelectric point. (Figures I-8 and I-9)

b. Preparation of Organisms

Bacillus subtilis spores were killed by the following treatments:

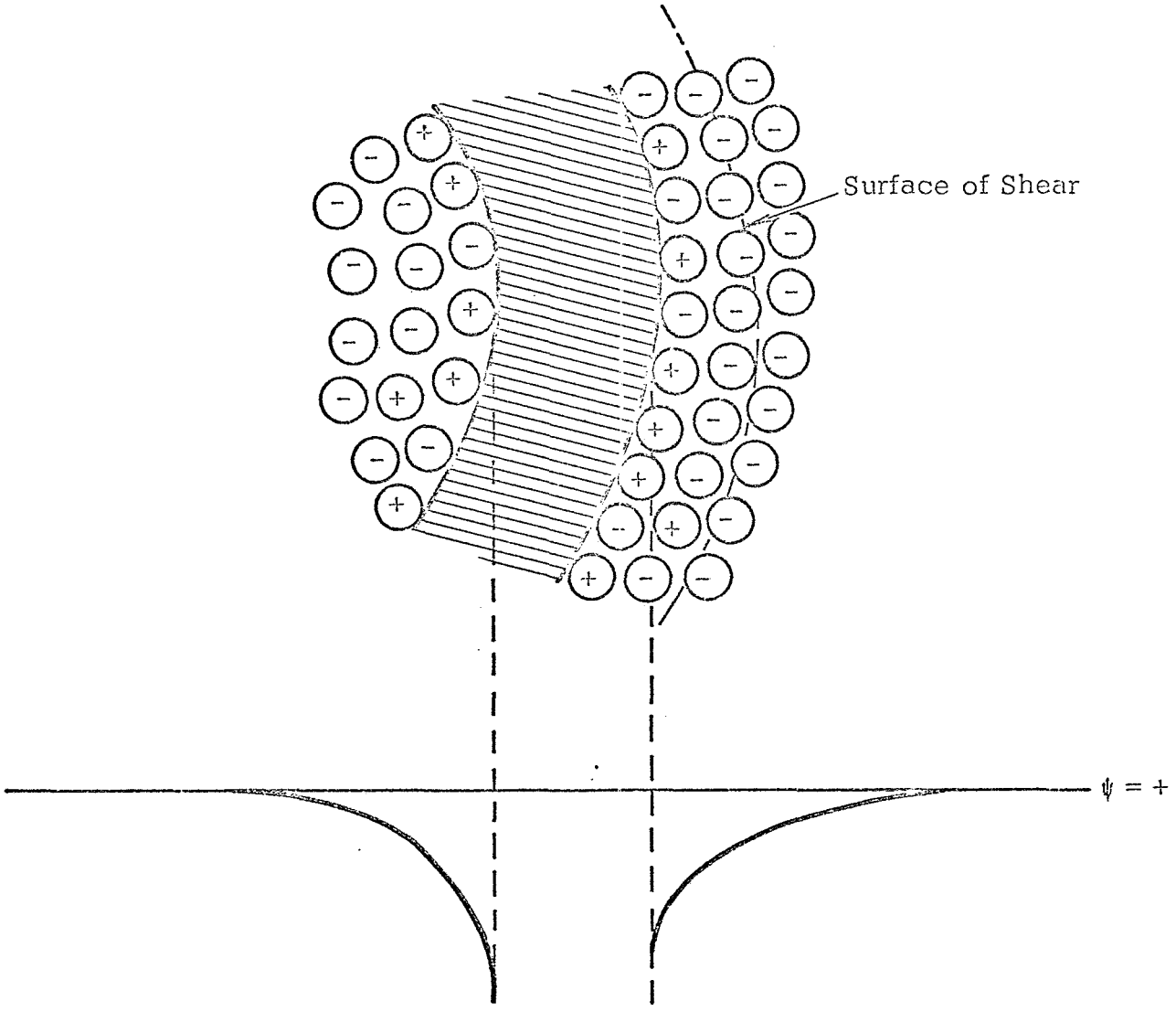


Figure I-8. Surface charge potentials are positive on both sides of the cell membrane. This occurs over a range of pH values in which amino groups are ionized and are more numerous than carboxyl or other acidic functions.

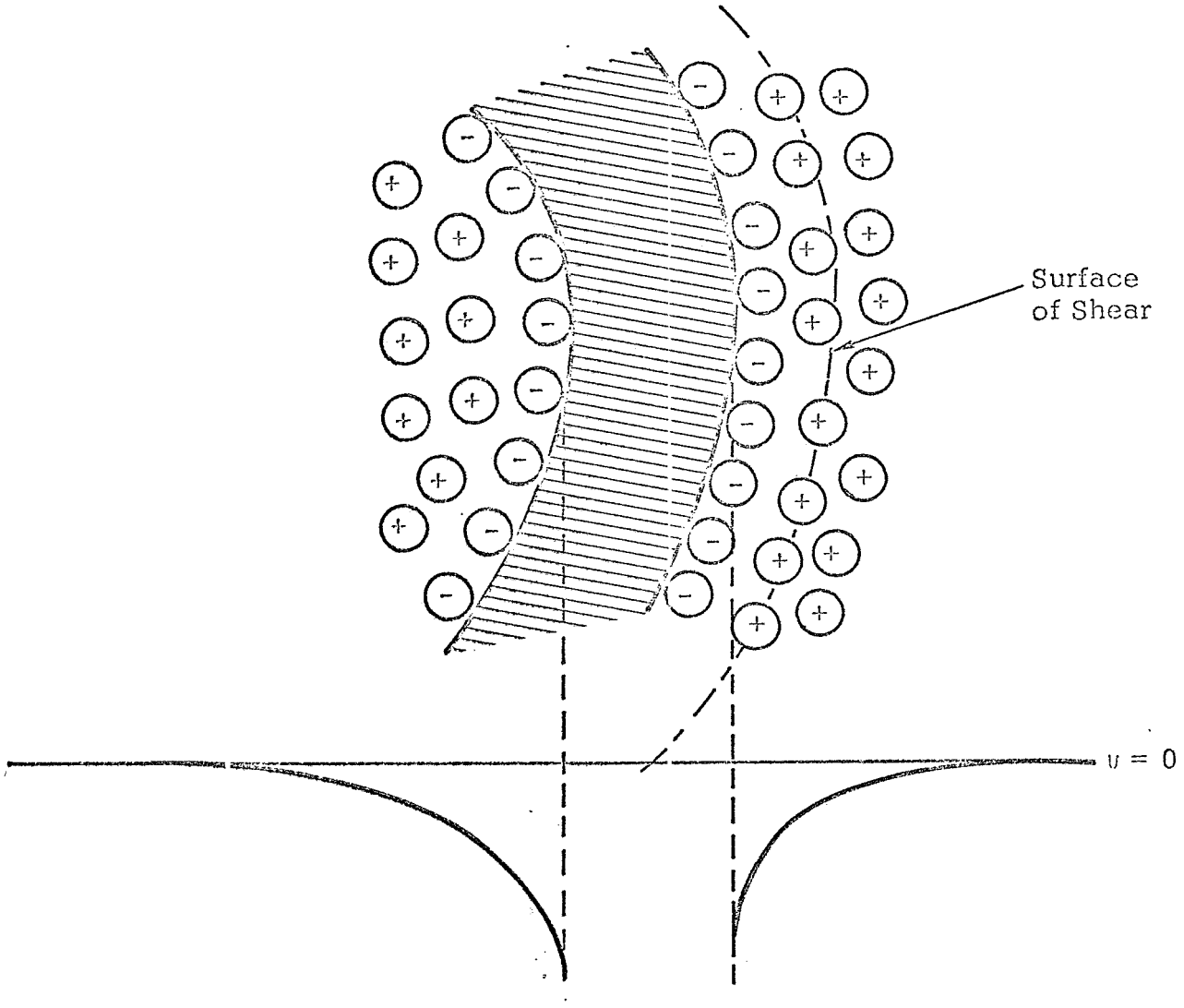


Figure I-9. The cancellation of surface charge potentials. Both sides of the cell membrane (including the cell wall) have an excess of negatively charged dissociable groups at high pH values when amino groups are not charged.

heating, exposure to formaldehyde fumes, to ethylene oxide and to chlorine. To insure that the above exposure treatments rendered the spores nonviable, samples of the suspensions were cultured on both Fluid Thioglycollate Media and Trypticase Soy. It was found that the treatments applied did induce sterility. The killed spore suspensions were used in the same way that the viable suspensions were used to determine what differences if any could be detected.

3. Results

The apparent isoelectric point of untreated B. subtilis spores was determined by microelectrophoresis to be 2.5. At pH ranges above and below 2.5 there were no reproducible differences between the viable and nonviable cells (Figures I-10 through I-14).

4. Conclusions

Electrophoresis of living and dead cell preparations did not show differences of sufficient order of magnitude to provide a useful means for differentiating between viable and nonviable cells. In order to provide a more sensitive means for the measurement of electrophoretic mobility as a function of pH it would be necessary to refine the technique. However, based upon the experiments performed upon the suspensions of B. subtilis, the order of magnitude of difference between viable and nonviable cells is so small that it is doubtful that even with highly refined apparatus that this method would show promise.

F. AUTORADIAGRAPHY

1. Introduction

A cell is metabolizing but not reproducing may in theory accumulate radioisotipe-labelled metabolites and thereby make itself radioactive. By use of the technique of autoradiography (Joftes 1963) these cells which have accumulated radioactive tracers may be visualized on a photographic plate (Figure I-15).

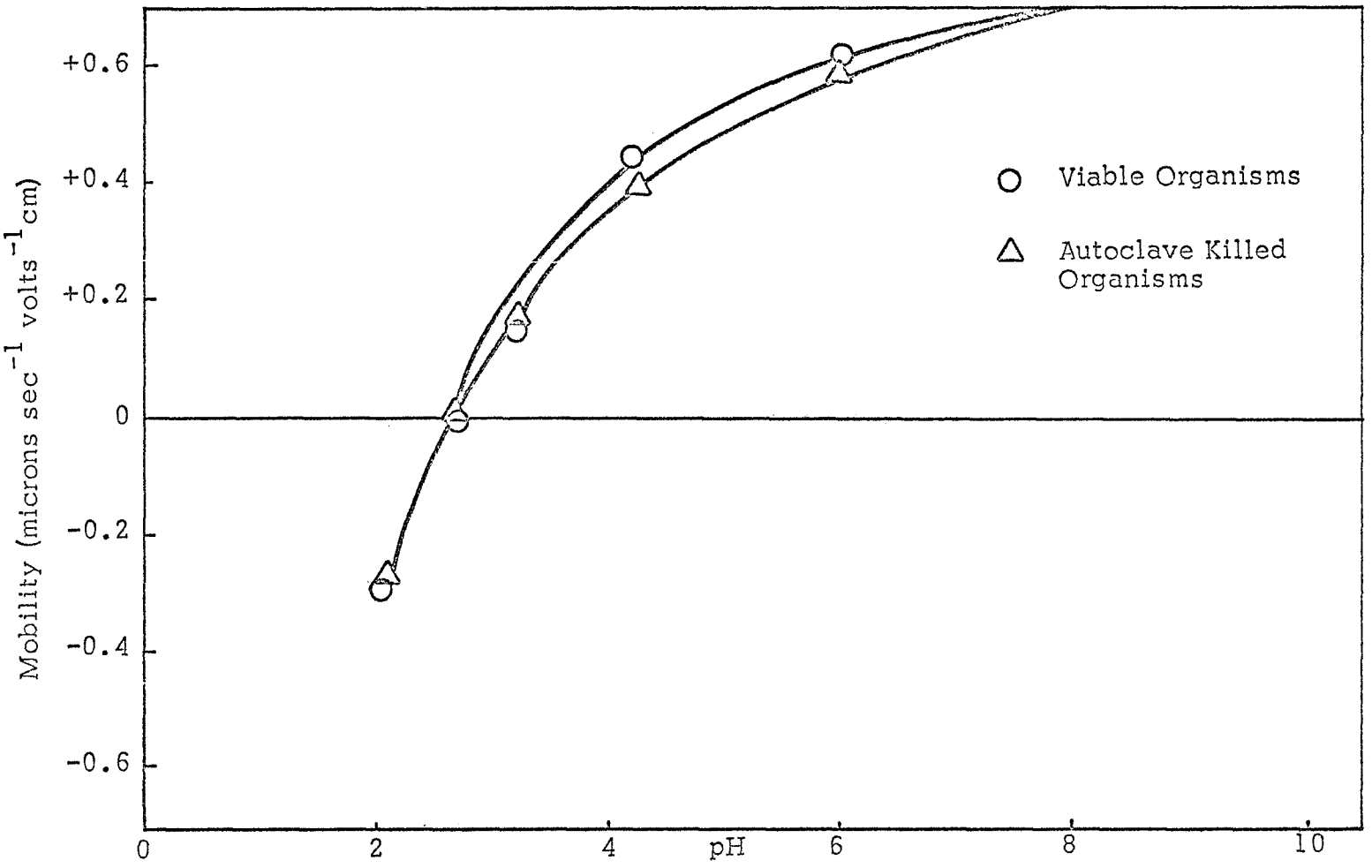


Figure I-10. The Effect of pH on the Mobility of Viable and Autoclave Killed Spores of B. subtilis var. niger

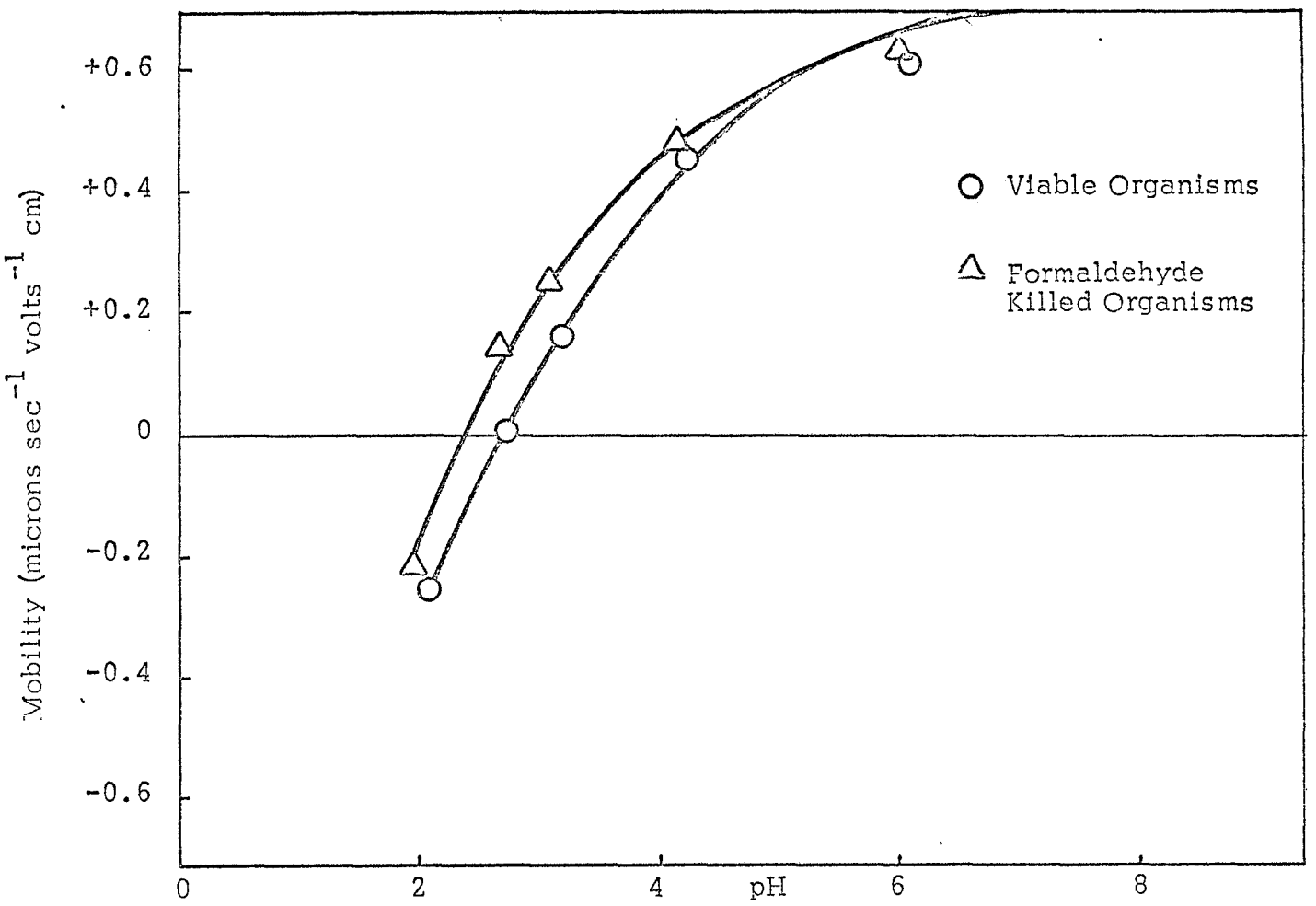


Figure I-11. The Effect of pH on the Mobility of Viable and Formaldehyde Killed Spores of B. subtilis var. niger

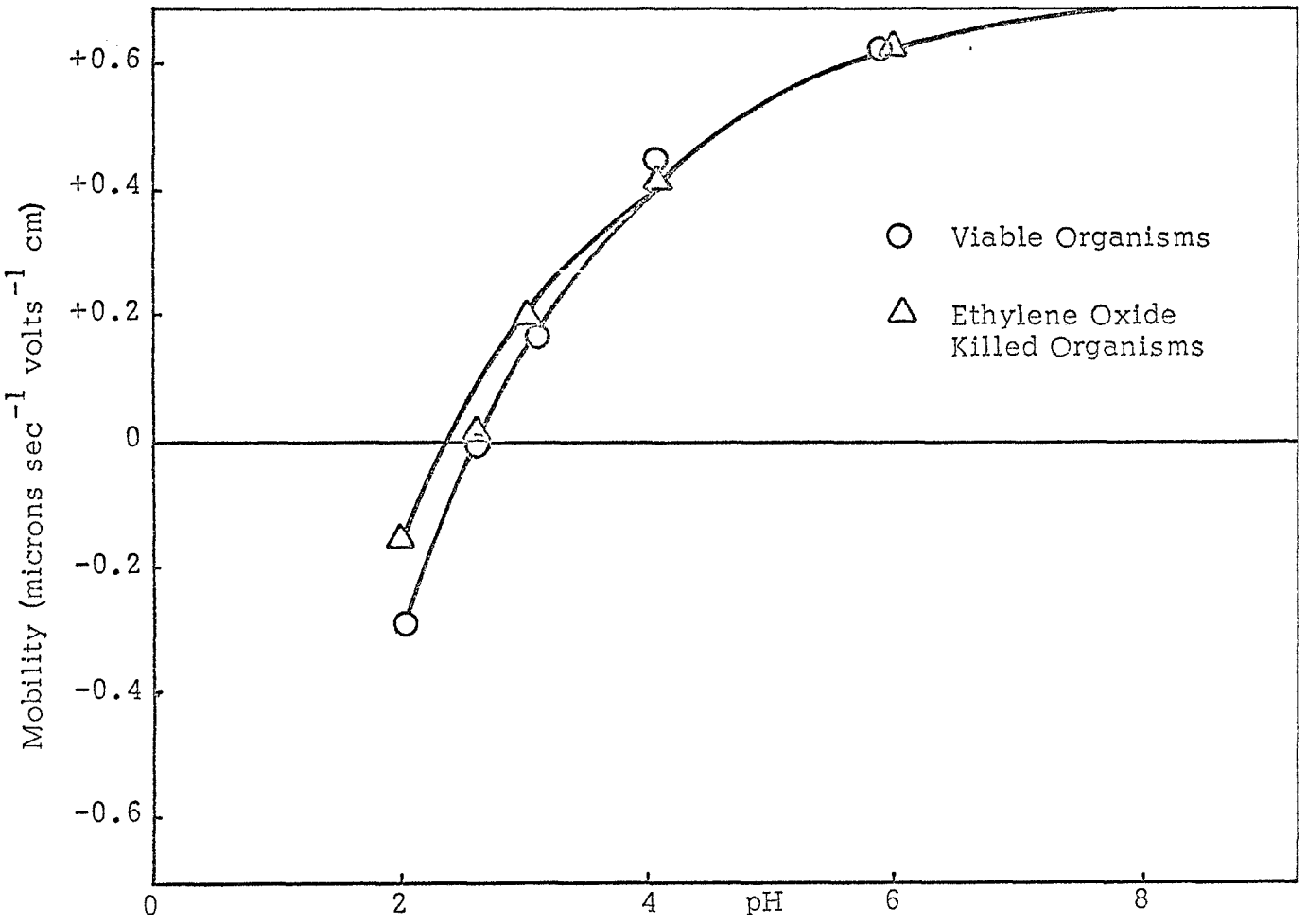


Figure I-12. The Effect of pH on the Mobility of Viable and Ethylene Oxide Killed Spores of B. subtilis var. niger

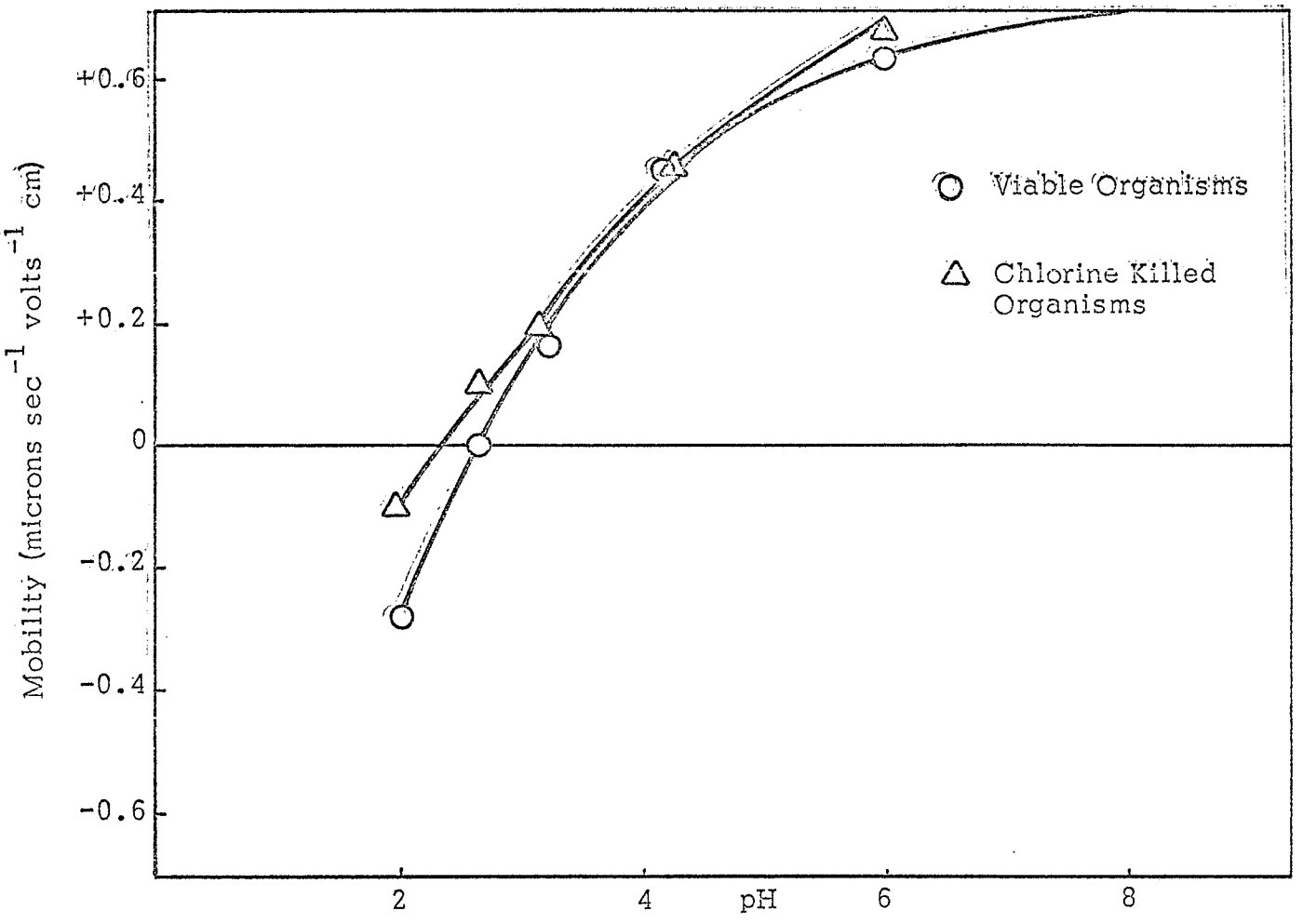


Figure I-13. The Effect of pH on the Mobility of Viable and Chlorine Killed Spores of B. subtilis var. niger

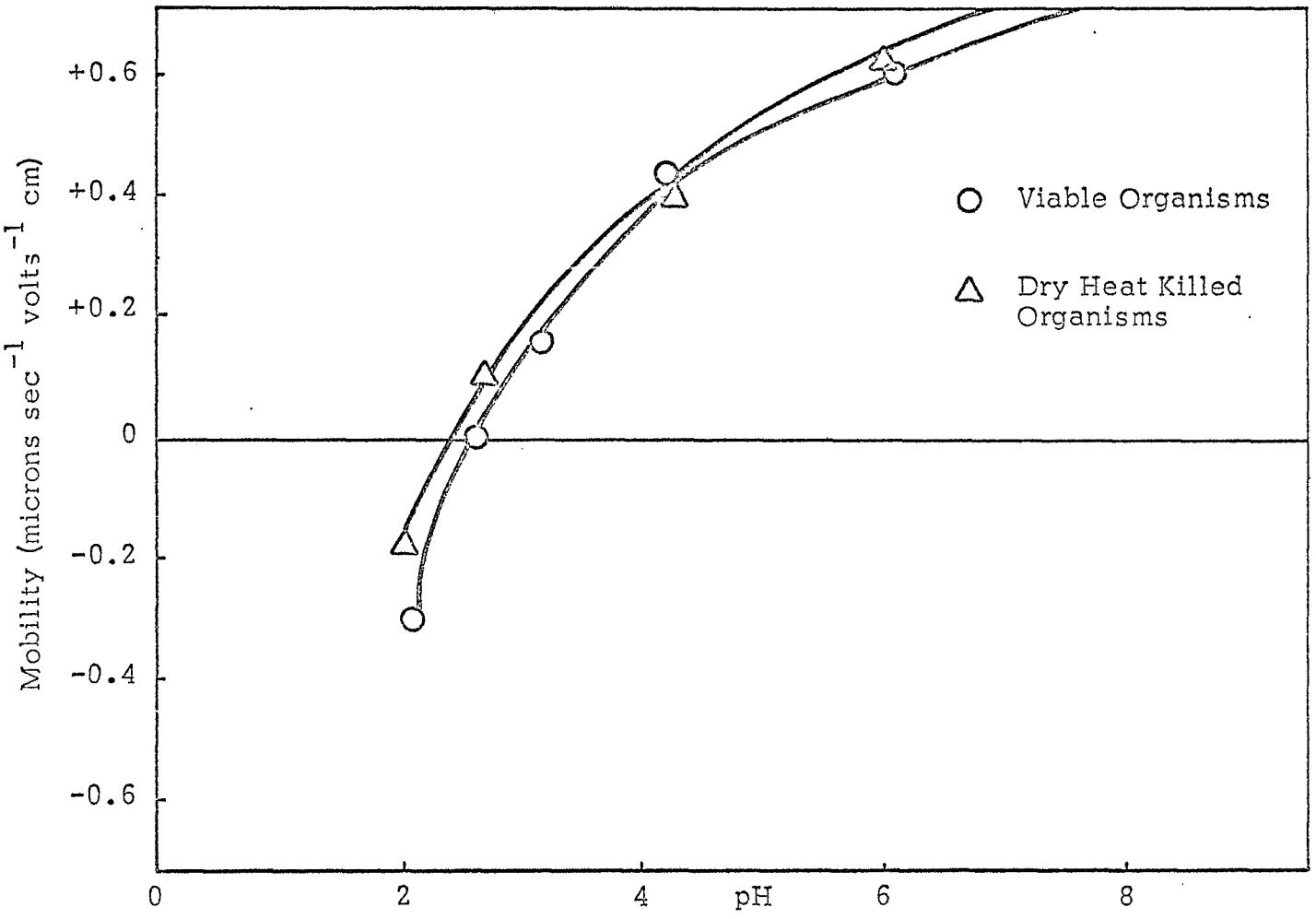


Figure I-14. The Effect of pH on the Mobility of Viable and Dry Heat Killed Spores of B. subtilis var. niger

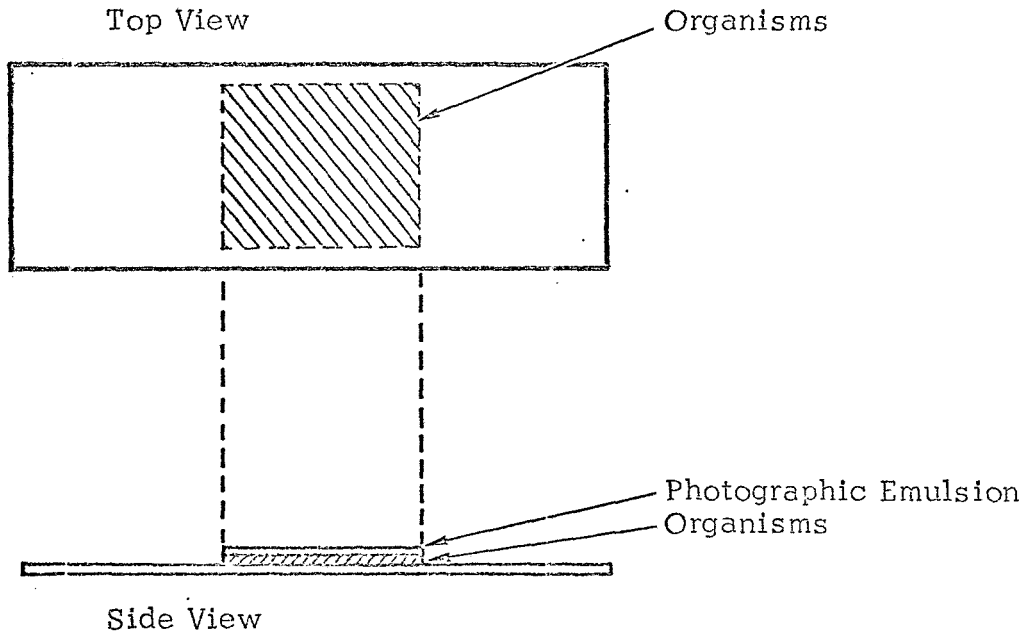


Figure I-15. Radio Autographs

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The principle of this method depends upon placing the organism rendered radioactive into intimate contact with a photographic emulsion which is sensitive to very weak beta radiation. The weak beta rays then strike silver crystals causing opaque "squiggles" at the site which is struck by the ray. The pattern of opaque silver on the emulsion after photographic development ideally should be similar to that of the shape of the microorganism. If the sample is stained, the silver grain should be observed within the boundary of the cell.

2. Methods

a. Organisms

Samples of viable spores of B. subtilis were killed by dry heat, autoclaving, exposure to ethylene oxide to ultraviolet irradiation, and to chlorine.

(1) Viable Spores. The viable spores were suspended in water to give a concentration of 10^8 cells per centrifuge tube (100 mg spores). The viable count of these spores was found to be 7.7×10^{10} viable spores per 100 mg.

(2) Heat Killed Spores. 100 mg of lyophilized spores (7.7×10^{10} organisms per centrifuge tube) were exposed to dry heat at 180°C for a period of 2 hours. 20 ml of sterile distilled water was added to the centrifuge tube.

(3) Ethylene Oxide Killed Spores. 7.7×10^{10} spores were exposed to ethylene oxide for a period of 8 hours at 40% relative humidity. They were suspended in 20 ml sterile distilled water.

(4) Autoclave Killed Spores. 7.7×10^{10} spores were autoclaved for 20 minutes at 15 lbs. pressure in 20 ml distilled water.

(5) Ultraviolet Killed Spores. 7.7×10^{10} spores distributed in a thin layer on a Petri dish were exposed in a sterile enclosure to an ultraviolet Sterilamp for a period of 4 hours at a distance of 6 inches.

(6) Chlorine Killed Spores. 7.7×10^{10} lyophilized spores were exposed to dry chlorine gas for a period of 2 hours. The organisms were then suspended in 20 ml of sterile distilled water.

G. DISCUSSION OF DETECTION METHODS

1. Electrophoresis

The nonculturing techniques studied did not provide a reliable means for distinguishing between living and dead cells. The electrophoretic behavior and staining characteristics are dependent upon charge distribution and surface characteristics of the cell walls of microorganisms. Figure I-7 shows the types of binding sites on cell walls. Dye molecules which possess electrical charges opposite to those present on the cell walls of microorganisms may lend selectivity to such organisms.

Electrophoretic methods for the separation of charged particles including microorganisms provide a means for separating such particles and organisms according to their electrophoretic mobility. In the studies conducted in which viable organisms were contrasted with nonviable organisms, the differences in electrophoretic mobility were extremely low and in some cases nonexistent. Thus, under the conditions of the experiments conducted it is clear that electrophoresis does not provide a reliable means of differentiating between viable and nonviable organisms. It is also apparent that in a complex mixture of solids particles and microorganisms that solid particles which possess a similar electrical charge distribution and may have the same or similar electrophoretic mobility as microorganisms and thus obscure attempts to diffentiate microorganisms from other solid debris.

2. Staining

While it is true that the binding or lack of binding of dyes to cell walls of microorganisms is not altered in a detectable way in nonviable as contrasted to viable microorganisms, the detection of organisms in either category is often helpful in assessing contamination levels. The presence of artifacts in stained samples of solids decrease the effectiveness of staining methods for the detection of viable or nonviable microorganisms.

The use of fluorescent staining techniques offers advantages over light microscopy staining techniques. The microscopic field background may

be so adjusted as to be essentially black. Microorganisms (living and dead) appear as brightly colored fluorescent structures against this background. Thus it is possible to microscopically scan since detection of a bright fluorescent object against a black background involves less fatigue and greater sensitivity since contrasting backgrounds do not appear when this method is ideally applied. Unfortunately, it is not possible to reliably differentiate between viable and nonviable organisms through the use of this method even under optimal circumstances since the differences between viable and nonviable cells involve differences in intensity of fluorescence.

3. Autoradiography

Autoradiography did not prove to be an effective method for the detection of microorganisms either viable or nonviable. The requirements inherent in the use of autoradiography are: (1) the cell must be permeable to the radioactive compound used and should retain the compound when washed, (2) the solid must be sufficiently uniform in texture such that less than 2.0 microns distance exists between organisms containing the radioactive label and the photographic emulsion, (3) that the solid does not nonspecifically bind the radioactive compound.

In the experiments conducted using radioautography, the prolonged time interval required even with large populations of spores would indicate that this method is relatively insensitive and time consuming. In addition if this method were to be applied to solids it would be almost impossible to pulverize solids uniformly without destroying the structural integrity of the organisms. Since the examination of the radioautograph is dependent upon the structural outlines of the cell, it is obvious that this method would be tedious and would lend itself to the detection of artifacts in addition to being insensitive.

4. Electron Spin Resonance

Electron spin resonance provides a means of detecting atoms or molecules possessing unpaired electrons (free radicals). Living systems contain enzymes which carry out biological oxidations and reductions which at some stage of electron transfer produce free radicals which may

be detected by the use of ESR resonance. Thus, it would appear that if only living systems contain free radicals, ESR spectroscopy would provide an elegant nondestrucive method for the detection of viable microorganisms in solid materials. Unfortunately, a large number of organic and inorganic substances exist which contain free radicals and which cannot be differentiated from the free radicals generated in living systems. Since the presence of variable quantities of free radicals of differing species may be demonstrated in many types of materials it does not appear that this tool would be useful for the detection of microbial contamination of solid materials.

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PHASE II. Freeing Microorganisms from Encasement

Certain representative solid materials were subjected to pulverization by drilling, ball mill, Waring blender, and mortar and pestle. The most useful of these methods of pulverization was found to be drilling. The viability of the microorganisms was established by culture methods. Attempts were likewise made to dissolve solids thereby freezing the microorganisms from the solid material. Very little difference was noticed whether organisms were freed from encasement by pulverization or by dissolving in a suitable solvent. It was established that certain of the solvents exerted toxic effects upon the microorganisms. The use of drilling without attempting to dissolve the solid was the pulverization procedure used in all subsequent experiments in Phases III and IV. The recoveries of B. subtilis var. niger were disappointingly low. In a typical experiment the solid contained $10^9 - 10^{10}$ microorganisms per gram. When 0.1 gram of the solid material was pulverized, only approximately 10^3 viable microorganisms could be demonstrated rather than an expected total count of $10^8 - 10^9$ microorganisms.

II. PHASE II. FREEING MICROORGANISMS FROM ENCASEMENT

A. INTRODUCTION

1. Statement of the Problem

Microorganisms may populate solids in significant numbers and yet be undetectable because the methods used for freeing them from encasement may cause a high percentage of them to be so damaged as to not survive the treatment. The particle size distribution of solids which may be optimal for small spores and bacteria, may not be suitable for application to large mycelial cells of fungi (Figure II-1). Some solids, because of their physical-chemical nature may be solubilized. Microorganisms contained within such solids may be detected by cultural methods if the solvent and/or other environmental efforts have not caused their destruction. The major problem in dissolving the solid is the rather small number of solids which may be solubilized without using combinations of heat, pressure and solvents which destroy microorganisms.

Plastics represent one of the largest categories of solids that may be contaminated with microorganisms. Information regarding the range of possible solvents and plasticizers which may be used for particular types of plastics have been discussed by Doolittle (1954).

In general, plastics which crosslink or which are thermosetting like epoxys are insoluble. Polyethylene is an exception to the class of thermosetting polymers. This plastic material will dissolve in xylene at temperatures over 50°C. Polystyrene dissolves in a variety of aromatic hydrocarbons at 25°C. These solvents are, however, toxic to most microorganisms (Snell 1954).

2. Theoretical Approaches

Pulverization represents another method of freeing microorganisms from encasement in solids. A wide variety of methods may be applied to reduce a solid to a state of subdivision satisfactory for cultural examination

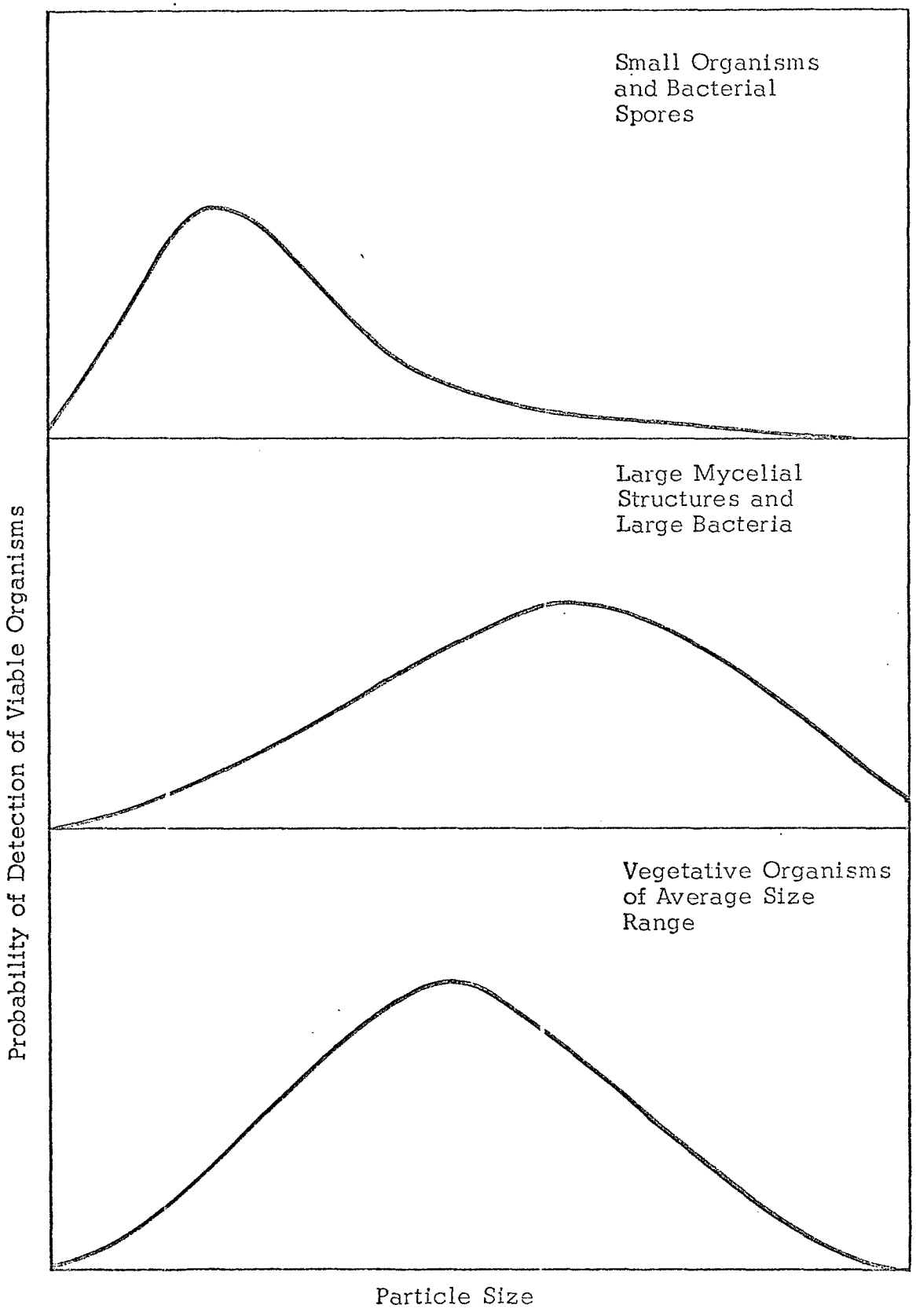
Small Organisms and Bacterial Spores
Large Mycelial Structures and Large Bacteria
Vegetative Organisms of Average Size Range
Probability of Detection of Viable Organisms
Particle Size

Figure II-1. Relationship between the Probability of the Detection of Viable Microorganisms in Solids and the Size of Particles

for microorganisms, The mortar and pestle has been used to pulverize various types of solids used as spacecraft components (Phillips 1960; Hoffman 1960). The latter technique relies upon crushing and shearing action. Morelli (1962) applied abrasion methods to pulverize a resin containing microorganisms added as inocula. A modification of this general method was also used by Portner (1962, 1963). Small pieces of solids may be pulverized by the use of crushing using a ball mill type of action. Mickle (1948) used this method for the disruption of materials. Ross (1963) describes a small efficient type of ball mill.

The use of the following methods of pulverization were investigated in Phase II for culturally recovering microorganisms added as inoculum:

(1) mortar and pestle
(2) ball mill
(3) Dremel drill
(4) Waring blender

Attempts were also made to solubilize the pulverized material prior to culturing.

B. METHODS

1. Organisms

Spores of B. subtilis were obtained as dry lyophilized spores from Ft. Detrick, Md. These spores were stored until needed at 5°C. When required for use as inocula, they were suspended in dilution bottles and assayed in Tryticase Soy Agar plates by decimal dilution. The number of culturally viable organism/gm was accurately established when inocula were prepared for addition to solids.

2. Materials

Paraplast is a hydrophobic material which is relatively soft, flexible and of moderate impact strength. It is easily sliced, producing large chips.

Parlodion is a cellulose nitrate derivative which has a hardness in excess of that measurable on the Shore A scale. It has high flexual and impact strengths. Its hardness makes it a difficult material to slice, or pulverize in a mortar and pestle; however, it may be drilled or pulverized in a ball mill and with considerable difficulty in a Waring blender.

Plaster of paris is a moderately hard, hydrophilic material possessing low flexual and impact strengths. It may be easily pulverized by all methods used in Phase II except the microtome. Attempts to slice this material with the latter instrument consistently lead to powdering and crumbling.

Eccocoat IC2 is a polyurethane coating material moderately hard with high flexual and impact strengths. The manufacturer describes it to be funginert, indicating that it is possibly toxic to some microorganisms.

3. Inoculation of Solids

The specimens were prepared with inocula of B. subtilis at levels indicated in Tables II-1 through II-4. No attempt was made to sterilize the materials before inoculating them. Because of the presence of B. subtilis spores in the laboratory where the work was performed the chance of accidental contamination could not be overlooked. The aluminum molds (Figure II-2) which were used in casting of the inoculated solids were autoclaved before use and sterile technique was used in the casting of the specimens. All materials were cured at room temperature. The inocula were added to the materials and mixed thoroughly just before the solid showed evidence of hardening. The inoculated solids were cured at room temperatures and maintained at room temperature until assayed.

4. Pulverization of Solids

With all of the methods of pulverization except the Dremel drill, the organism was cooled to liquid nitrogen temperature just before pulverization. In the case where drilling was used, the sample was drilled while immersed in autoclave sterilized culture media for a period of 1/2 minute.

The mortar and pestle were used inside a decontaminated polyethylene bag. The solid was pulverized for two minutes and transferred to sterile Trypticase Soy Agar plates and cultured.

The blender vessel (one quart capacity) was autoclaved. The solid was added and pulverized for one minute at top speed. It was rinsed from the blender bowl and transferred to the Trypticase Agar plates.

5. Cultural Assay

A limit exists to the size of population of any particular species of microorganism which can be assayed on a single agar surface. As colonies become more numerous, the frequency of two or more cells lying so closely together that their colonies overlap increases. The colonies which develop early deplete the agar of nutrient and thereby suppress the development of visible colonies from cells which start proliferation later.

Trypticase soy agar plates were chosen for the assay medium because of their ability to produce a high degree of germination and colony development in spores of B. subtilis. They also permit easy quantitative measurement of the proportion of the inoculum recovered. The program at Phase II was not really addressed to precise measurement of recovery percentages in the easy cases but was addressed to the development of a method of promise for measuring the internal populations of spores in materials difficult to test for sterility.

6. Determination of Particle Size

Each of the various solids was encased in the aluminum molds in the same manner indicated in Figure II-2. The samples were subjected to drilling while immersed in culture media. The particles were removed from solution by centrifugation at 2000 x g for 1 hour in order to sediment the finest particle size present. The sediment was resuspended in a small volume of distilled water and examined microscopically. 500 particles were counted and the results are shown in Figures II-3 through II-6.

The samples were also subjected to pulverization using the ball mill, mortar and pestle, and Waring blender. With the exception of plaster of

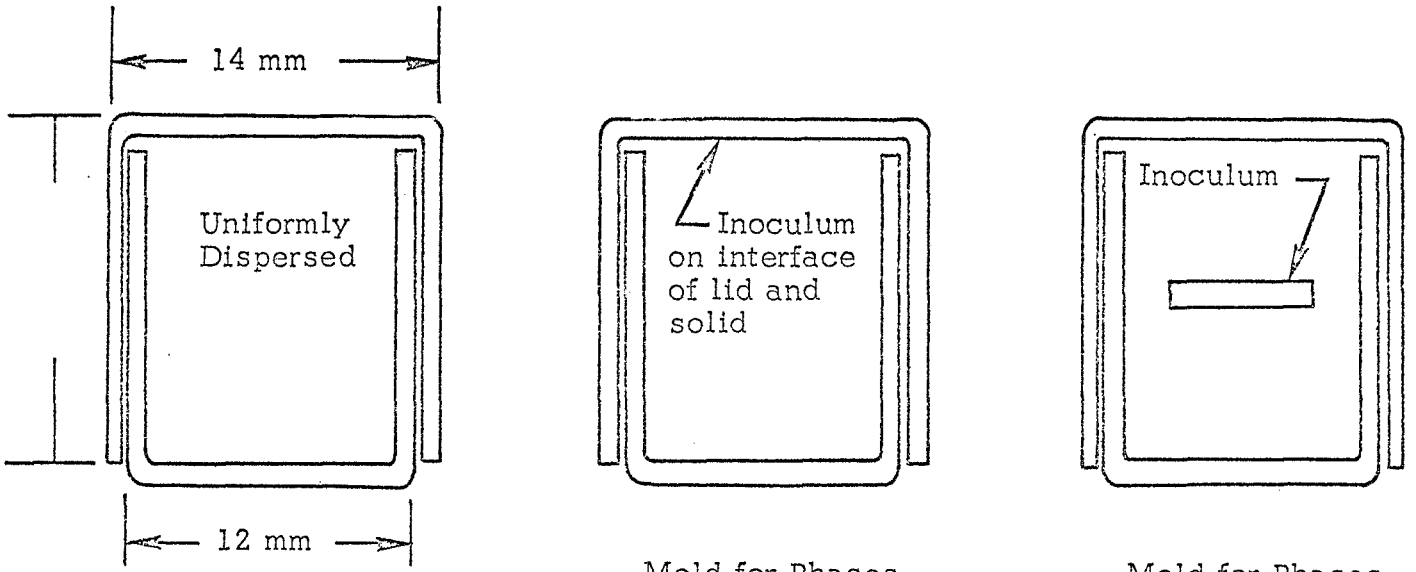

Figure II-2. Locations in Inocula in the Solid Materials

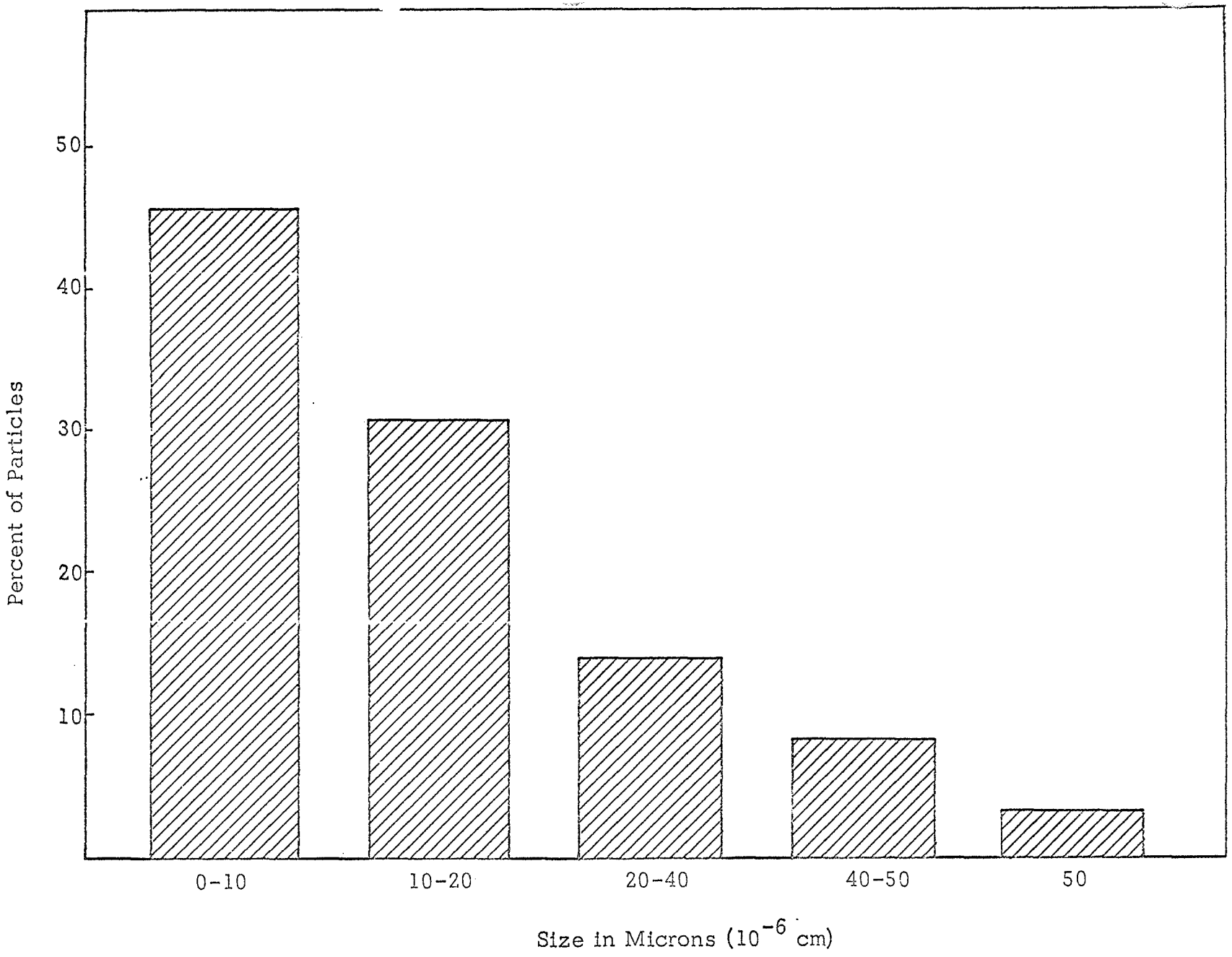

Figure II-3. Particle Size Distribution of Plaster of Paris Pulverized by Drilling

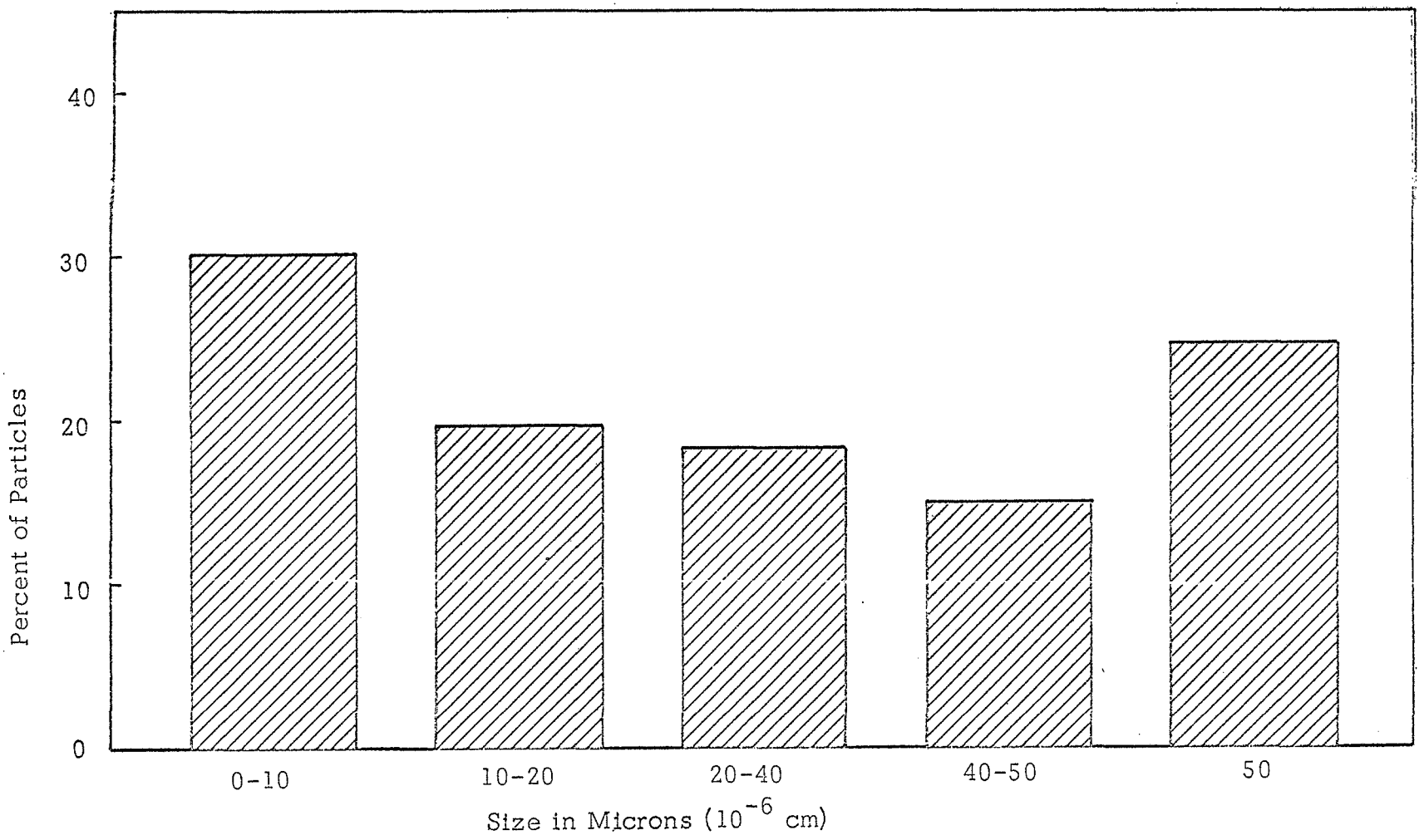

Figure II-4. Particle Size Distribution of Parlodion Pulverized by Drilling

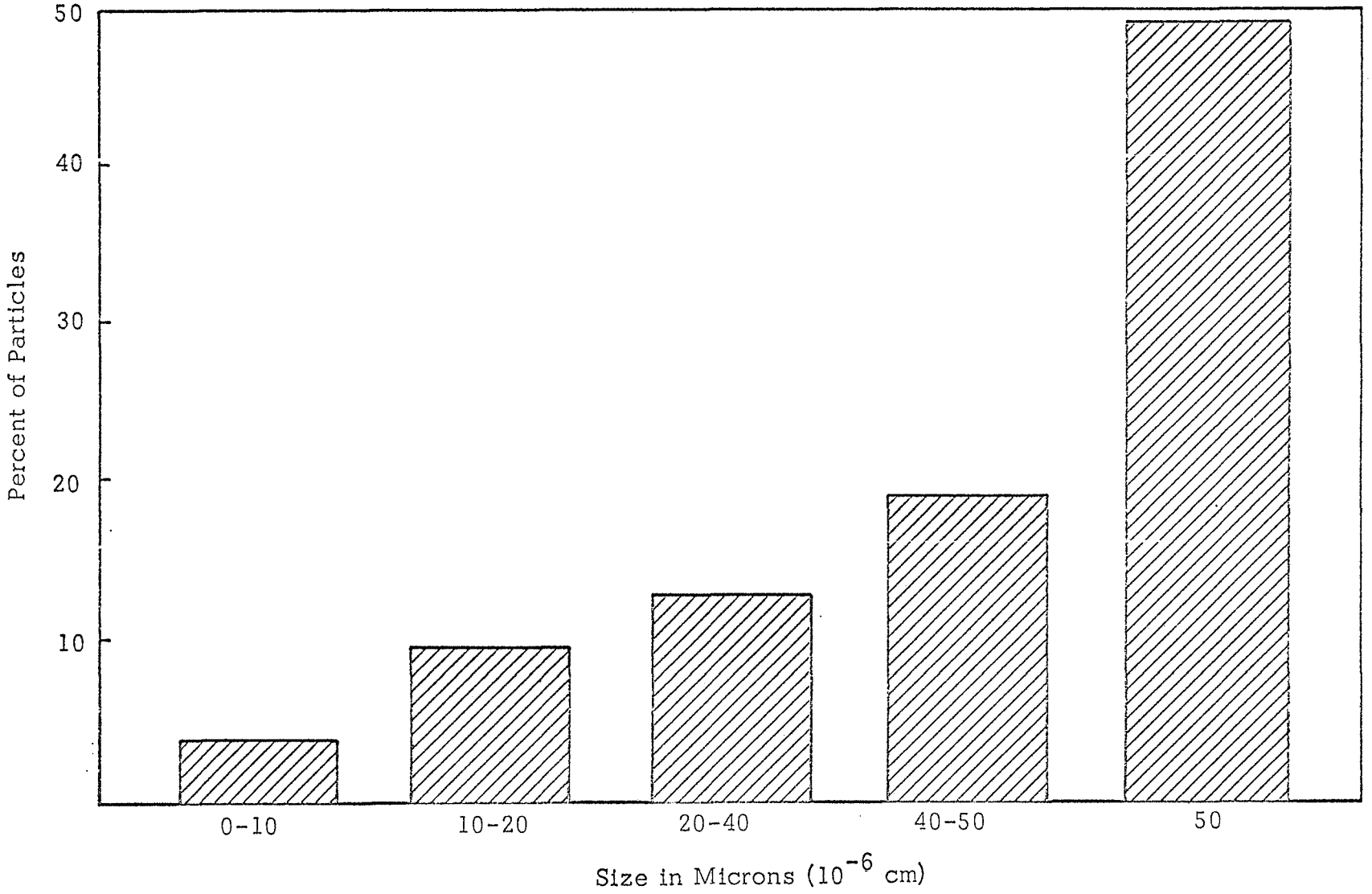

Figure II-5. Particle Size Distribution of Stycast 2651 Pulverized by Drilling

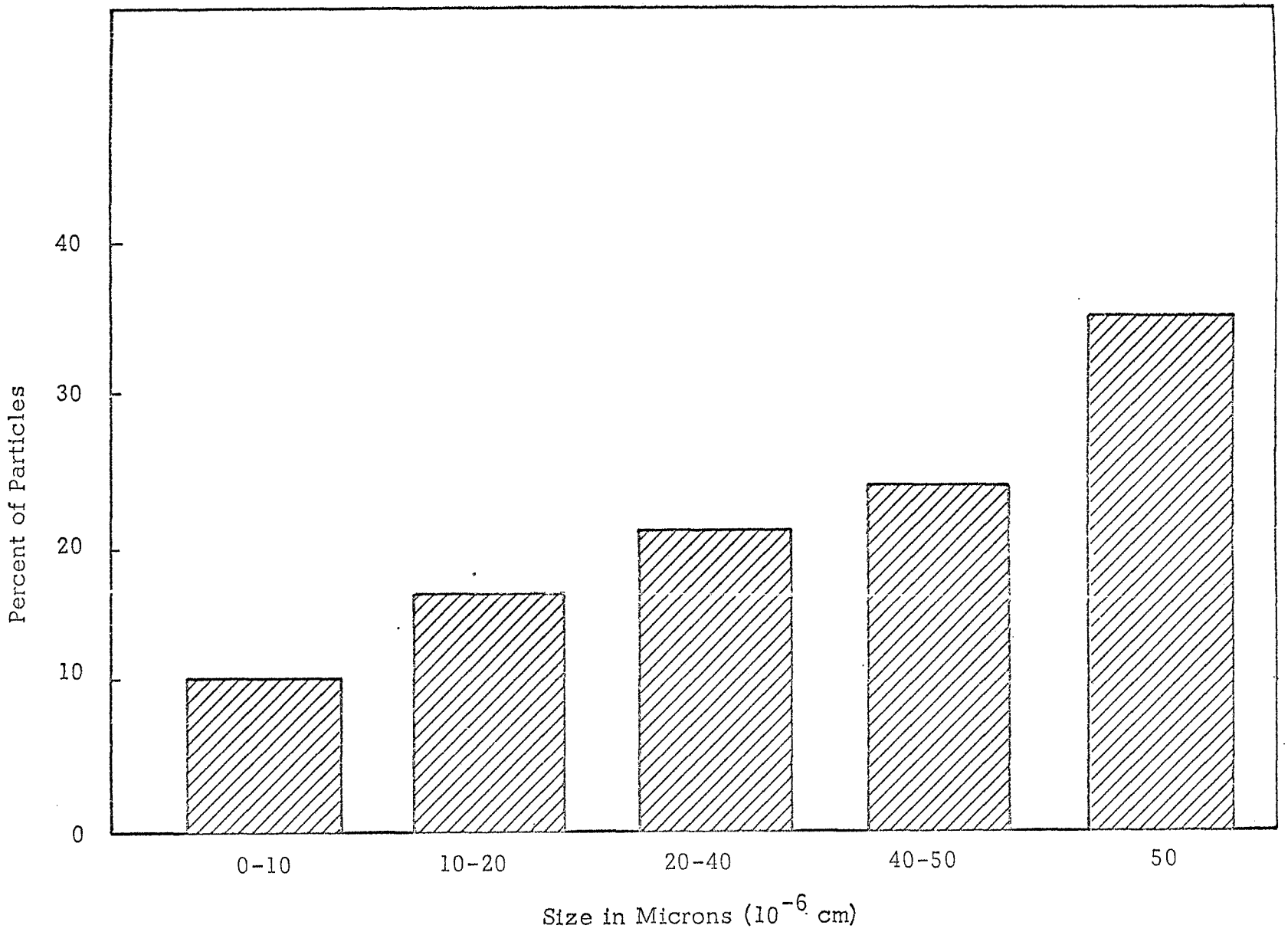

Figure II-6. Particle Size Distribution of Piccotex 120 Pulverized by Drilling

paris, none of the above methods produced satisfactory pulverization. The plaster of paris could be fragmented to particles less than 10 microns; however, the range of distribution of particle sizes varied considerably. The Waring blender pulverized the parlodion particles after preliminary freezing with liquid nitrogen. Unfortunately, however, as the particle sizes were reduced below 25 microns, the resultant aerosol ignited and exploded.

For paraplast (wax) one ml of the acetone suspension of its particles from Specimen No. 2 was diluted 10 to 1 in xylene and an aliquot of the resulting solution was used to inoculate the test agar.

One ml of the water suspension of parlodion particles was dried in an aluminum dish and the residue was dissolved in 10 ml of acetone. An aliquot of the resulting solution was placed on the test agar.

One ml of the acetone suspension of plaster of paris particles was diluted to 10 to 1 in 3% ethylene diamine tetraacetic acid (EDTA) solution. Again an aliquot of the resulting solution was assayed for viable spores.

One ml of the water suspension of Eccocoat IC2 particles was dried in an aluminum dish. The residue was dissolved in dimethyl formamide and the resulting solution was assayed for viable spores.

7. Experimental Design of Recovery Experiments

For each of the four materials and each of the pulverizing processes three inoculated specimens were prepared. These specimens were processed in the following way:

Specimen No.	Pulverized	Cultured Directly	Cultured After Dissolving Solid Material
1	yes	yes	no
2	yes	no	yes
3	no	no	yes

Specimen No. 1 indicated the overall recovery portion for the process and material. Specimen No. 2 (compared with No. 1) showed how many spores were freed when the particles of solid were dissolved away from them. Specimen No. 3 permitted measurement of the toxic and bacteriostatic effects of the solid material and the solvent on the inoculum.

C. RESULTS

Recovery of viable organisms from the solids tested indicated that the drill provided the most effective means of pulverization compatible with culturing methods. The mortar and pestle, blender, and ball mill allowed recovery of smaller number of B. subtilis (Tables II-1 through II-4).

Obviously, the solids from which microorganisms may be most easily recovered are paraplast and plaster of paris (Tables II-1 and II-3). Recovery from parlodion and Eccocoat IC2 were consistently more difficult than the former two solids (Tables II-1 through II-4).

TABLE II-1

EFFECT OF VARIOUS METHODS OF PULVERIZATION AND SOLUBILIZATION ON THE RECOVERY OF BACILLUS SUBTILIS VAR. NIGER FROM PARAPLAST

Treatment of Sample	Inoculum (Organisms/ gm Solid)	Theoretical No. Colonies Based on Size of Inoculum	Actual No. of Colonies Observed			
			Drill	Ball Mill	Mortar and Pestle	Blender
1. Pulverized	2×10^8	10^8	6×10^3	5×10^2	5×10^3	2×10^3
2. Pulverized and Solubilized	2×10^8	10^8	4×10^3	3×10^3	5×10^3	8×10^3
3. Solubilized	2×10^8	10^8	6.5×10^3	--	--	--

TABLE II-2

EFFECT OF VARIOUS METHODS OF PULVERIZATION AND SOLUBILIZATION ON THE RECOVERY OF BACILLUS SUBTILIS VAR. NIGER FROM PARLODION

Treatment of Sample	Inoculum (Organisms/ gm Solid)	Theoretical No. Colonies Based on Size of Inoculum	Actual No. of Colonies Observed			
			Drill	Ball Mill	Mortar and Pestle	Blender
1. Pulverized	10^9/gm	5×10^7	2×10^2	Less than 10	10^2	50
2. Pulverized and Dissolved	10^9/gm	5×10^7	10^3	Less than 10	30	30
3. Dissolved	10^9/gm	5×10^7	9×10^3	--	--	--

TABLE II-3

EFFECT OF VARIOUS METHODS OF PULVERIZATION AND SOLUBILIZATION ON THE RECOVERY OF BACILLUS SUBTILIS VAR. NIGER FROM PLASTER OF PARIS

Treatment of Sample	Inoculum (Organisms/ gm Solid)	Theoretical No. Colonies Based on Size of Inoculum	Actual No. of Colonies Observed			
			Drill	Ball Mill	Mortar and Pestle	Blender
1. Pulverized	10^9	2×10^8	10^4	2×10^3	5×10^3	5×10^3
2. Pulverized and Dissolved	10^9	2×10^8	5×10^3	7×10^3	8×10^3	9×10^3
3. Dissolved	10^9	2×10^8	7×10^3	--	--	--

TABLE II-4

EFFECT OF VARIOUS METHODS OF PULVERIZATION AND SOLUBILIZATION ON THE RECOVERY OF *BACILLUS SUBTILIS* VAR. *NIGER* FROM ECCOCOAT IC2

Treatment of Sample	Inoculum (Organisms/ gm/Solid)	Theoretical No. Colonies Based on Size of Inoculum	Actual No. of Colonies Observed			
			Drill	Ball Mill	Mortar and Pestle	Blender
1. Pulverized	10^9	2×10^8	5×10^2	Less than 10	5×10^2	Less than 10
2. Pulverized and Dissolved	10^9	2×10^8	5×10^2	Less than 10	Less than 10	10
3. Dissolved	10^9	2×10^8	8×10^3	--	--	--

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PHASE III. Demonstration of the Applicability of Drilling Techniques for Recovery of Microorganisms in Solids

A variety of solid materials were inoculated at three different locations with spores of B. subtilis var. niger, Clostridium sporogenes, and Ulocladium. The distribution of the inocula included the following: uniformly distributed throughout applied to the interface between the solid and the aluminum lid, and at the interface of an aluminum disc and the solid material. The microorganisms were exposed from the solid materials by the use of drilling techniques. The recoveries of microorganisms in all cases were extremely low. When the inoculum was uniformly distributed throughout the solid, the recoveries were higher than when the inoculum was applied at either of the two interfaces.

The solubility of the various solids in a variety of solvents were studied in Phase III. The toxicity of various solvents was determined.

III. PHASE III . DEMONSTRATION OF THE APPLICABILITY OF DRILLING TECHNIQUES FOR RECOVERY OF MICROORGANISMS IN SOLIDS

A. INTRODUCTION

The objective of Phase III concerns the demonstration of the applicability of the best method (drilling) in Phase II to several solid materials of different physical properties. The solid materials investigated in this phase includes those with a wide range of physical and chemical characteristics. The solids are representative of those used as potting compounds, plastic structural components, dielectrics, and coatings.

B. METHODS

1. Preparation of Solid Materials

The solid materials investigated are listed in Tables III-1 through III-4. All materials were mixed with the catalyst in the recommended ratios and the inoculum of microorganisms was added to the material just prior to its showing evidence of solidifying. The dental cups which are composed of soft aluminum alloy were autoclaved prior to filling with the solid materials. The three locations of the inocula in relation to the dental crown containers used as receptacles are shown in Figure III-1. The procedures for culturing drilled specimens are shown on Table III-5. The isolator, specimen, and drilling assembly is shown in Figure III-1.

2. Sectioning of Propellant

The solid propellant was cast with the inoculum at Jet Propulsion Laboratory, cured, and submitted to Dynamic Science. This material was not treated in the same manner as the other solids, but was sliced with a blade-type microtome and subsequently cultured. The microtome procedure involved the use of an adjustable device which advanced the propellant to the blade cutting surface. Slices of 400 microns were

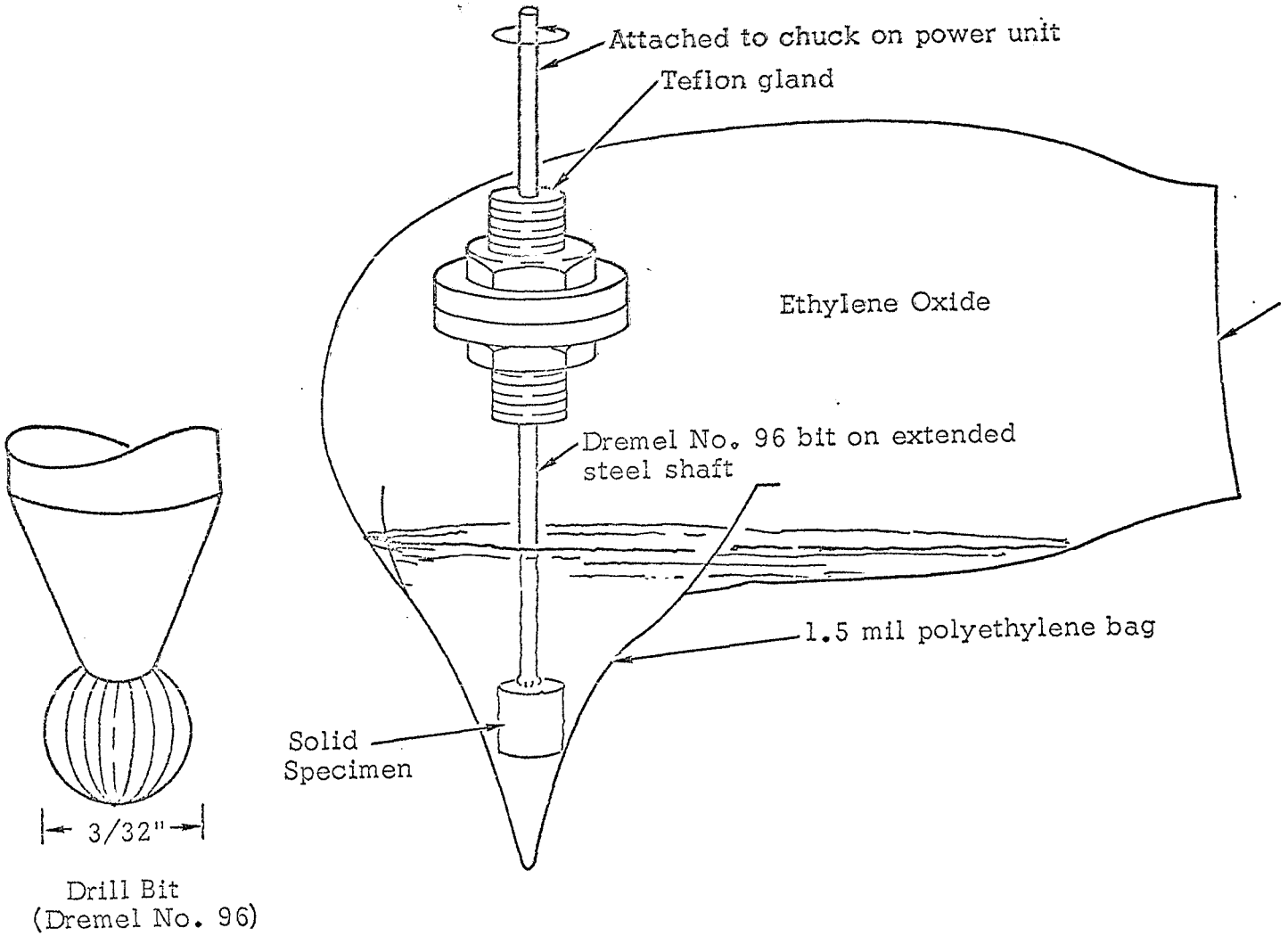


Figure III-1. Isolator, Specimen and Drilling Assembly

TABLE III-1

PROPERTIES OF VARIOUS SOLIDS STUDIED IN PHASES II AND III

Name of Principal Constituent	Common Use	Properties					
		Principal Constituent	Hardness, Shore A	Flex. Str. lb/in^2	Impact Str. ft lb/in of notch	Density g/ml	Water abs. %
Paraplast Parlodion	dental casting many	paraffin cellulose nitrate	55 - 60				
Plaster of Paris	many	calcium sulfate	97				
Eccocoat IC2	coating	polyurethane	80				
Piccotex 120	casting resin	polystyrene	99				
Stycast 2850GT	casting resin	epoxy resin	90 - 95	13,300	0.24	2.0	0.1
RTV-40	potting compound	Silicone rubber	55			1.37	
Stycast 2651 MM	potting compound	epoxy polymer	92 - 97	12,000	0.2		0.15

TABLE III-2

PROPERTIES OF VARIOUS SOLIDS STUDIED IN PHASE III

Name of Principal Constituent	Common Use	Properties					
		Principal Constituent	Hardness, Shore A	Flex. Str. lb/in^2	Impact Str. ft lb/in of notch	Density g/ml	Water abs. %
Epon 901/B3	adhesive	epoxy polymer	92 -97				
Eccogel 1265	adhesive	epoxy gel	25			1.0	
Propellant	solid propellant	ammonium perchlorate and polyurethane	70				
RTV-40	foaming resin	silicone rubber foam	43 - 47				
Stycast 1090	foaming resin	epoxy foam	85 - 90	4200		0.78	0.1
CAT-A-LAC 443-1-500	coating coating		85 - 90 Rex				
Eccocoat IC2	coating	polyurethane	80				

TABLE III-3

SOURCES OF MATERIAL

Material	Manufacturer or Supplier	Address
Epon 901/B-3	Shell Chemical Company	
Eccogel 1265	Emerson and Cuming, Inc.	Canton, Mass.
Propellant	Jet Propulsion Laboratory	Pasadena, Calif.
RTV-40	General Electric Company Silicone Products Department	Waterford, N. Y.
Stycast 1090	Emerson and Cuming, Inc.	Canton, Mass.
CAT-A-LAC 443-1-500	Finch Paint and Chemical Company	Torrance, Calif.
Eccocoat IC2	Emerson and Cuming, Inc.	Canton, Mass.

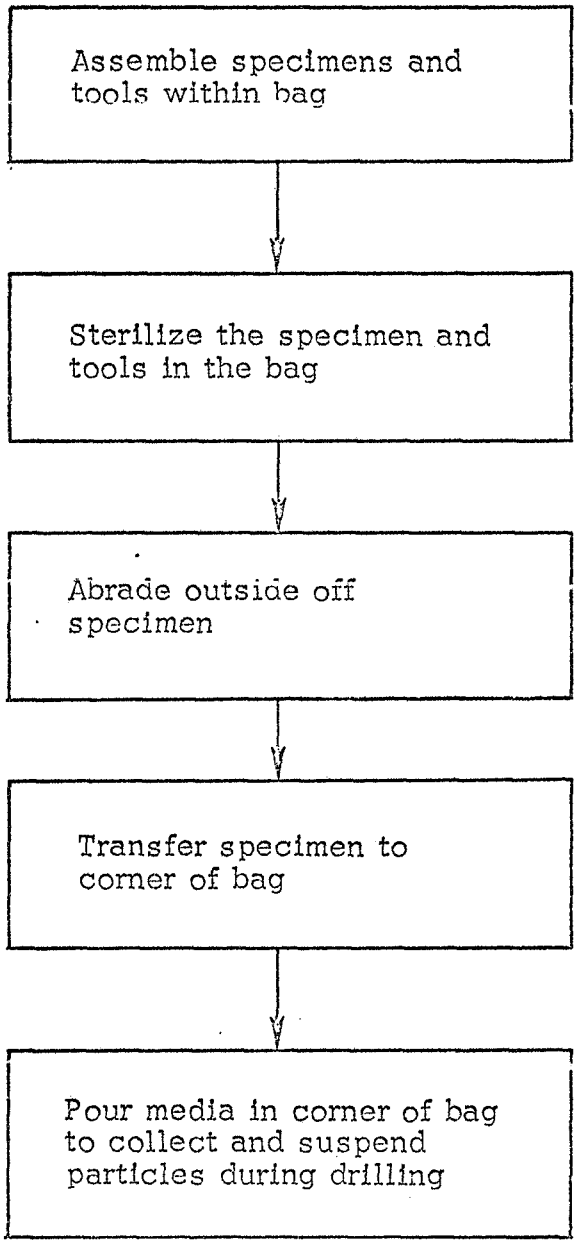
TABLE III-4

SOURCES OF MATERIAL

Material	Manufacturer or Supplier	Address
Paraplast	Brunswick Corporation	St. Louis, Mo.
Parlodion	Mallinkrodt Chemical Company	St. Louis, Mo.
Plaster of Paris	------	-----
Eccocoat IC2	Emerson and Cuming, Inc.	Canton, Mass.
Piccotex 120	Pennsylvania Industrial Chemical Corporation	Philadelphia, Pa.
Stycast 2850GT	Emerson and Cuming, Inc.	Canton, Mass.
RTV-40	General Electric Company Silicone Products Department	Waterford, N. Y.
Stycast 2651MM	Emerson and Cuming, Inc.	Canton, Mass.

TABLE III-5

PROCEDURE FOR CULTURING DRILLED SPECIMENS


Assemble specimens and tools within bag
Sterilize the specimen and tools in the bag
Abrade outside off specimen
Transfer specimen to corner of bag
Pour media in corner of bag to collect and suspend particles during drilling

possible with the use of this device. Below this thickness, the slices fragmented and were discontinuous.

The sections were approximately 3 x 10 cm^2. The core from which the propellant samples were taken was prepared by sterilizing the outer surfaces of the propellant with ethylene oxide vapor in polyethylene gas sterilant bags (Figure III-1). The ethylene oxide and water vapor concentrations were established through the use of gas chromatography. Attempts were made to pulverize propellant with the use of drilling techniques. Unfortunately, the cut fragments and chips adhered to each other and produced and agglomerated dense mass of material rather than dispersible particles.

Attempts were made to drill propellant which had previously been frozen in liquid nitrogen. Freezing, however, did not sufficiently alter the physical characteristics of the propellant such that pulverization in the absence of agglomeration was possible. Earlier work with propellant demonstrated that it may be pulverized by abrading with emery paper. However, appreciable loss would take place because the propellant would clog the abrasive on the paper and render it useless. Another method which was tried and found to have more promise than those previously used was abrading with a stickle back rasp.

The organisms studied in Phase III were B. subtilis, Clostridium sporogenes, Ulocladium, and Staphlococcus epidermidis. The organisms were prepared in mixed inocula, suspended in 0.01 ml of redistilled water and added to the ingredients of the solid materials just prior to the ingredients showing evidence of hardening. All materials in Phase III were inoculated as described except propellant. The total weight by Celite used for each level of mixed inoculum ranged between 2 and 3 grams. The Celite (Johns Mansville) was previously sterilized in a dry oven at 170°C for sixteen hours. The contents of the Celite organism inocula were added to 1000 gm propellant formula by personnel at Jet Propulsion Laboratory.

3. Preparation and Assay of Inoculum

The organisms studied in Phase III were B. subtilis, Clostridium sporogenes, Ulocladium, and Staphlococcus epidermidis.

a. Bacillus subtilis

Spores of this organism were obtained from Fort Detrick in the lyophilized state. These organisms were suspended in dilution vials and viability counts performed following decimal dilution by plate counting techniques within one day of their use as inocula in solids.

b. Clostridium sporogenes

Of the anaerobic spore formers, only Clostridium sporogenes (the National Canners Assoc. P.A. -3679) was sufficiently nonpathogenic to be convenient for the purpose of this program. The confined environment of cells encased within plastic solids suggests that spores might survive this type of anaerobic environment. The Clostridial spores were prepared by growing vegetative organisms in Brain Heart Infusion Broth (DIFCO). Calcium chloride was added as a supplement to the media at a final concentration of 1.0% to enhance sporulation. The organisms were harvested by centrifugation at approximately 1000 x g and the sediment was examined microscopically to insure that they were in the form of spores. The supernatant fluid was discarded and the spores were washed three times by alternately centrifuging and resuspending in sterile distilled water. The washed suspended cells were subjected to exposure to heat at 80°C for a period of 10 minutes to destroy surviving vegetative forms and to insure that only the spores survive. Following this heat shock step, the cells were stored at 5°C until required as an inoculum. In order to provide the inoculum at specific levels for JPL, the cells were assayed by decimal dilution in anaerobic deep agar tubes and by the use of Brewer Anaerobic Agar (BBL) plates.

c. Ulocladium

Spores of the fungus Ulocladium were originally isolated from dust obtained from the JPL assembly facilities. The fungus was identified as

Ulocladium by Dr. Emory G. Simmons, Head of the Mycology Laboratory of the U. S. Army Natick Laboratories. This fungal organism is most similar to Alternaria consortiale (Thum) Grove and Hughes. Although this organism resembles both Alternaria and Stemphylium, Ulocladium shows morphological differences.

The Ulocladium was grown on Sabouraud Agar medium (DIFCO). These organisms were harvested at the end of the first week of growth in distilled water and were subjected to sonication in an Acoustica Associates Model DR50 AH ultrasonic bath for a period of 20 minutes at 40 to 60 mca at a power output which produced cavitation. Following sonication the cells were centrifuged at 1000 x g for 10 minutes, the supernatant fluid discarded and resuspended in sterile distilled water. This procedure was repeated twice. The cells were then resuspended in sterile distilled water in a sterile dilution bottle and maintained at 5°C until required for use as an inoculum. At the time of submission of the organism to Jet Propulsion Laboratory, plate counts on Sabouraud Agar medium (DIFCO) were performed.

4. Measuring Solubility and Toxicity

The solubility of the various solids is indicated in Tables III-6, 7, and 8. The toxicity of the various solvents on the growth of B. subtilis is shown in Tables II-9 and 10.

C. RESULTS

Recovery of B. subtilis was obtained in certain instances whereas Clostridium sporogenes and Ulocladium were not recovered (Tables III-11 through 18). When the inoculum was distributed uniformly throughout the solid, recovery levels were superior to those obtained when the inoculum existed either at the interface of the lid and the solid or at the juncture of the aluminum disc and the solid. Rate of growth of spores of B. subtilis was depressed when grown in the presence of the various constituents of the solid plastics materials (Tables III-9 and 10). It is not surprising that organic solvents are generally toxic to spores of B. subtilis since some of these solvents are used to disrupt the cell membranes of microorganisms in biochemical enzyme studies.

TABLE III-6

SOLUBILITY OF SOLIDS IN VARIOUS SOLVENTS

S = Soluble; I = Insoluble

Solid Material	Solvent			
	Ethylene Diamine	Nitrilo Triethanol	Dimethyl Formamide	Carbon Disulfide
Eccocoat IC2	S	I	S	I
Cat-A-Lac	I	I	I	I
Stycast 1090	I	I	I	I
Epon 901	S	I	I	I
Stycast 2651	I	I	I	I
RTV-40	I	I	I	I
Stycast 2850	I	I	I	I

TABLE III-7

SOLUBILITY OF SOLIDS IN VARIOUS SOLVENTS

S = Soluble; I = Insoluble; C = Colloidal

Solid Material	Solvent			
	Glacial Acetic	Pyridine	Tetra-Hydrofuran	Turpentine
Eccocoat IC2	I	I	S	I
Cat-A-Lac	I	C	C	I
Stycast 1090	I	I	I	I
Eccogel 1265	I	S	I	I
Epon 901	C	S	I	I
Stycast 2651	I	I	I	I
RTV-40	I	I	I	I
Stycast 2850	I	I	I	I

TABLE III-8

SOLUBILITY OF SOLIDS IN VARIOUS SOLVENTS

PS = Partial Soluble; I = Insoluble

Solid Material	Solvent			
	Toluene	Benzene	Methanol	Glycerin
Eccocoat IC2	PS	PS	I	I
Cat-A-Lac	I	I	I	I
Stycast 1090	I	I	I	I
Epon 901	I	I	I	I
Stycast 2651	I	I	I	I
RTV-40	I	I	I	I
Stycast 2850	I	I	I	I

TABLE III-9

TOXICITY OF VARIOUS SOLVENTS TO B. SUBTILIS VAR. NIGER IN TRYPTICASE SOY BROTH

+ = Growth; - = No Growth

Solvent	Spore Conc.	Time Elapsed					
		15 hr	16 hr	19 hr	21 hr	23 hr	39 hr
Benzene	10^6	+	+	+	+	+	+
Toluene	10^6	-	-	+	+	+	+
Tetra Hydrofuran	10^6	-	-	+	+	+	+
Tetraethylene Pentamine	10^6	-	-	-	-	-	-
Ethylene Diamine	10^6	-	-	-	-	-	-
Dimethyl Formamaide	10^6	-	-	+	+	+	+
Pyridine	10^6	+	+	+	+	+	+

TABLE III-10

TOXICITY OF MATERIALS TO B. SUBTILIS VAR. NIGER IN TRYPTICASE SOY BROTH

+ = Amount of Growth; - = No Growth

Material Analyzed	Spore Conc.	Time Elapsed Before Detachable Growth					
		5 hr	16 hr	19 hr	21 hr	23 hr	39 hr
LIQUIDS:							
Control Culture	10^6	+	++	++	+++	++++	++++
Cat-A-Lac	10^6	-	-	-	-	-	+
Catalyst 443-1-500	10^6	0	+	+	+	+	+
Eccogel 1265A	10^6	-	-	-	-	+	+
Eccogel 1265B	10^6	-	-	-	-	-	-
Epon B-1	10^6	-	-	-	-	-	-
Epon B-3	10^6	-	-	-	-	-	-
Eccocoat IC2-A	10^6	-	-	-	-	-	+
Eccocoat IC2-B	10^6	-	-	-	-	-	-
Diluent IC2	10^6	-	-	-	-	+	+
Catalyst 9	10^6	-	-	-	-	-	-
Catalyst 11	10^6	-	-	-	-	-	+
Acetone	10^6	-	-	+	+	+	+
SOLIDS:							
Epon 901	10^6	-	-	-	-	-	+
Stycast 2850	10^6	-	-	-	-	-	+
Stycast 1090	10^6	-	-	-	-	-	+
Stycast 2651	10^6	-	-	-	-	-	+
RTV-40	10^6	-	-	-	-	-	+

TABLE III-11

DETECTION OF MICROORGANISMS UNIFORMLY INOCULATED THROUGHOUT VARIOUS SOLIDS (PULVERIZED BY DRILLING)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminants

Level of Inoculum	Solids Tested				
	Piccotex 120	Stycast 2850 GT	RTV-40	Stycast 2651	Epon 901
0	0/5	0/5	0/5	0/5	2/5
10	0/5	0/5	0/5	1/5C	0/5
10^2	5/5C	0/5	0/5	0/5	0/5
10^4	0/5	0/5	5/5B	0/5	0/5
10^6	0/5	0/5	0/5	1BC/5	5B/5

TABLE III-12

DETECTION OF MICROORGANISMS UNIFORMLY INOCULATED THROUGHOUT VARIOUS SOLIDS (PULVERIZED BY DRILLING)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminants

Level of Inoculum	Solids Tested				
	Eccogel 1265/A	Stycast 1090	RTV-40+10% RTV 921	Cat-A-Lac	Eccocoat IC2/A
0	0/5	0/5	0/5	0/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10^2	0/5	0/5	0/5	0/5	0/5
10^4	0/5	0/5	0/5	0/5	0/5
10^6	0/5	0/5	0/5	0/5	0/5

TABLE III-13

DETECTION OF MICROORGANISMS UNIFORMLY INOCULATED THROUGHOUT VARIOUS SOLIDS (ORGANISMS FREED FROM SOLID BY DISSOLVING IN APPROPRIATE SOLVENT)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminants
- because of no suitable solvent assays not performed

Level of Inoculum	Solids Tested				
	Piccotex 120	Stycast 2850 GT	RTV-40	Stycast 2651	Epon 901
0	-	-	-	-	0/5
10	-	-	-	-	0/5
10^2	-	-	-	-	0/5
10^4	-	-	-	-	0/5
10^6	-	-	-	-	0/5

TABLE III-14

DETECTION OF MICROORGANISMS UNIFORMLY INOCULATED THROUGHOUT VARIOUS SOLIDS (ORGANISMS FREED FROM SOLID BY DISSOLVING IN APPROPRIATE SOLVENT)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminants

\- because of no suitable solvent assays not performed

Level of Inoculum	Solids Tested				
	Eccogel 1265/A	Stycast 1090	RTV 40 + 10% RTV 921	Cat-A-Lac	Eccocoat IC2/A
0	0/5	-	-	-	0/5
10	0/5	-	-	-	0/5
10^2	0/5	-	-	-	0/5
10^4	0/5	-	-	-	1BC/5
10^6	0/5	-	-	-	1BC/5

TABLE III-15

DETECTION OF MICROORGANISMS AT AN INTERFACE WITH THE ALUMINUM CAP AND THE SOLID (SITE OF INOCULUM WAS EXPOSED TO CULTURE MEDIA)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminant

Level of Inoculum	Solid Tested				
	Piccotex 120	Stycast 2850 GT	RTV 40	Stycast 2651	Epon 901
0	0/5	0/5	0/5	0/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10^2	0/5	0/5	0/5	0/5	1B/5
10^4	0/5	0/5	0/5	0/5	0/5
10^6	0/5	0/5	0/5	0/5	0/5

TABLE III-16

DETECTION OF MICROORGANISMS AT AN INTERFACE WITH THE ALUMINUM CAP AND THE SOLID (SITE OF INOCULUM WAS EXPOSED TO CULTURE MEDIA)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminant

Level of Inoculum	Solids Tested				
	Eccogel 1265/A	Stycast 1090	RTV 40 + 10% RTV 921	Cat-A-Lac	Eccocoat IC2/A
0	0/5	0/5	0/5	0/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10^2	0/5	1B/5	0/5	0/5	0/5
10^4	0/5	0/5	0/5	0/5	0/5
10^6	0/5	0/5	0/5	0/5	0/5

TABLE III-17

DETECTION OF MICROORGANISMS AT AN INTERFACE BETWEEN AN IMBEDDED ALUMINUM DISC AND THE SURROUNDING SOLID MATERIAL (PULVERIZED BY DRILLING)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminant

Level of Inoculum	Solids Tested				
	Piccotex 120	Stycast 2850 GT	RTV 40	Stycast 2651	Epon 901
0	0/5	0/5	0/5	5C/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10^2	0/5	0/5	0/5	0/5	0/5
10^4	0/5	1B/5	0/5	0/5	5B/5
10^6	0/5	0/5	0/5	0/5	3B1C/5

TABLE III-18

DETECTION OF MICROORGANISMS AT AN INTERFACE BETWEEN AN IMBEDDED ALUMINUM DISC AND THE SURROUNDING SOLID MATERIAL (PULVERIZED BY DRILLING)

B = B. subtilis var. niger; S = Cl. sporogenes; U = Ulocladium; C = Contaminant

Level of Inoculum	Solids Tested				
	Eccogel 1265/A	Stycast 1090	RTV 40 + 10% RTV 921	Cat-A-Lac	Eccocoat IC2/A
0	0/5	0/5	0/5	0/5	0/5
10	0/5	0/5	0/5	0/5	0/5
10^2	0/5	1C/5	0/5	1C/5	1C/5
10^4	0/5	3B/5	0/5	0/5	0/5
10^6	0/5	5B/5	0/5	0/5	0/5

D. DISCUSSION

The recoveries of microorganisms from solids studied were all quite low. In part these results were due to the growth inhibitory effects of the solvents used in the solubilization of the solids. Freezing and thawing microorganisms cause damage to cell walls. The extent of this effect was not independently determined. The pulverization methods also had an effect upon the recovery of microorganisms. The Dremel drill appears to be the most useful of the methods studied. The shearing and cutting forces of this method of pulverization seem to be less injurious than the crushing effects of the ball mill or the crushing, shearing effects of the mortar and pestle. Unfortunately, there is no indication as to whether the relatively low recoveries were also due in part to the production of local heat. It would appear that heat, pressure and cutting forces of the Dremel drill may cause this method to be far from optimal as a method of pulverization even though it provided the best recoveries of the various methods tested.

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PHASE IV. Determination of the Reliability of Recovery of Microorganisms from Solid Materials by Means of Drilling Technique

The drilling and culturing techniques found most suitable in Task III were tested for reliability in Task IV. The reliability of recovery of inoculated spores of B. subtilis var. niger was studied utilizing seventy-five cultural determinations at 37°C and an equal number at 25°C. The recoveries were highest when the inoculum was distributed uniformly throughout the solid and lowest when the inoculum was distributed at the interface of either the aluminum disc or lid. The recoveries utilizing drilling and cultural techniques have been disappointingly low and are likely due to a number of factors which are discussed in the body of this report and in the Addendum.

IV. PHASE IV. DETERMINATION OF THE RELIABILITY OF RECOVERY OF MICROORGANISMS FROM SOLID MATERIALS BY MEANS OF DRILLING TECHNIQUE

A. INTRODUCTION

The objective of Phase IV is to establish the reliability of recovery of microorganisms inoculated into selected solid materials. Seventy-five determinations were attempted with each material in order to provide a statistically reliable number of trials.

The reliability of the recovery methods established in Phase III using the drilling technique and the inoculum sites are previously discussed in Phase III and illustrated in Figure II-1 was established. In place of using a mixed inoculum in this experiment, spores of B. subtilis were the only microorganisms studied.

B. METHODS

The organisms were grown and assayed as described in Phase III. All assays were performed within one day of employing the organisms as inocula. The inocula were incorporated with the materials tested in the same location and manner as previously discussed in Phase III.

In order to avoid systematic errors relating to the sequence in which analyses were performed, all three forms of inoculum distribution were used in daily analyses. Every attempt was made to maintain the same technique and in particular to control the rate and extent of pulverization of the samples. It was noted, however, that local heating took place during the course of drilling. Although the exact amount of heating that took place was not determined, its presence was nevertheless detected.

C. RESULTS

The recovery of organisms introduced as inocula into the various samples are reported in Tables IV-1, IV-2, IV-3. The largest number of microorganisms were recovered from solids in which B. subtilis spores were uniformly distributed throughout the solid. The smallest number of

TABLE IV-1

DETERMINATION OF RELIABILITY OF ASSAY SENSITIVITY

SAMPLES OF EACH SOLID TESTED TO DETERMINE REPRODUCIBILITY OF RECOVERY OF 6×10^8 SPORES *B. subtilis*/Gm UNIFORMLY DISTRIBUTED THROUGHOUT SOLID

	Tube Assay		Plate Assay	
Material	37°C	25°C	37°C	25°C
Stycast 2850 GT	0/75	0/75	0/75	0/75
RTV-40	1/75	0/75	3/75	0/75
Epon 901/B3	4/75	5/75	2/75	4/75
Stycast 1090	0/75	0/75	0/75	0/75
Eccocoat IC2	30/75	9/75	12/75	9/75

TABLE IV-2

DETERMINATION OF RELIABILITY OF ASSAY SENSITIVITY

SAMPLES OF EACH SOLID TESTED TO DETERMINE REPRODUCIBILITY OF RECOVERY OF 6×10^8 SPORES *B. subtilis*/Gm INOCULUM AT INTERFACE OF LID AND SOLID

	Tube Assay		Plate Assay	
Material	37°C	25°C	37°C	25°C
Stycast 2850 GT	0/75	0/75	0/75	0/75
RTV-40	2/75	0/75	1/75	0/75
Epon 901/B3	5/75	0/75	0/75	1/75
Stycast 1090	0/75	0/75	0/75	3/75
Eccocoat IC2	6/75	3/75	5/75	0/75

TABLE IV-3

DETERMINATION OF RELIABILITY OF ASSAY SENSITIVITY

SAMPLES OF EACH SOLID TESTED TO DETERMINE REPRODUCIBILITY OF RECOVERY OF 6×10^8 SPORES *B. subtilis* /Gm INOCULUM LOCATED AT INTERFACE OF ALUMINUM DISC AND SOLID

	Tube Assay		Plate Assay	
Material	37°C	25°C	37°C	25°C
Stycast 2850 GT	2/75	1/75	0/75	0/75
RTV-40	0/75	0/75	1/75	0/75
Epon 901/B3	0/75	1/75	2/75	1/75
Stycast 1090	0/75	1/75	0/75	0/75
Eccocoat IC2	3/75	0/75	2/75	1/75

microorganisms recovered was when the inoculum was located at the interface of the aluminum disc and the solid (Table IV-3). The growth of microorganisms was more rapid at 37°C than at 25°C. In most instances the tube assay appeared to be superior in recovery to the plate assay.

D. DISCUSSION

It is apparent that the recoveries of sizable inocula by the use of drilling techniques were disappointingly low. The possible reasons for such low recoveries may be manifold. In the course of pulverizing the samples the organisms residing at the various locations in the solids may have been ineffectively exposed. This appears to be likely in view of the fact that the particle distribution of plastic materials similar in nature to those studied indicated that the ideal particle size was neither uniform nor sufficiently small to free the encased organisms. Furthermore, in attempting to reconcile these results with those found later in our laboratory, it was discovered that the particle size distribution obtained during pulverization of plastic materials varied as a function of the pressure applied while drilling, the sharpness of the drill, and the operator.

The temperature rise during the course of drilling varied according to the area being drilled. The distribution of the inocula included the following: uniformly distributed throughout, applied to the interface between the solid and the aluminum lid, and at the interface of an aluminum disc and the solid material.

Recent experiments conducted by the authors to establish why recoveries of large populations of microorganisms were so disappointingly low, indicate that in addition to pulverization parameters, the presence of toxic materials within the plastic and in some instances the toxicity of the solvents play extremely significant roles. Some of our more recent work on solids in which attempts have been made to minimize these effects are discussed in the Addendum which follows.

E. CONCLUSIONS

Regardless of the site of inoculum, whether uniformly mixed throughout the solid, applied at an interface between the solid and the oid, or applied at the interface of an aluminum disc and the solid, the recovery of microorganisms used as inocula was uniformly unsatisfactory. There appeared to be differences in the recoveries of microorganisms from the various solids. In addition, it appeared that best recoveries were obtained when the microorganisms were distributed uniformly throughout the solid. In the instances in which the microorganisms were deposited at the interface between the lid and the solid, and between the face of the aluminum disc and the solid, the recoveries were considerably lower.

ADDENDUM

The interim between the completion of the work reported in Phases I through IV of this program and the writing of this addendum, improvements on the dilution methods for microorganisms in solid materials have been made. These have involved extension in the range of sensitivity of detecting viable microorganisms to 10^2 microorganisms per cm^3 of solid material and in some instances to as low as 10 microorganism/cm^3.

The improvements in detection first took place in the study of microorganisms in solid propellants. While conducting these investigations it became apparent that certain modifications must be made in the recovery techniques in order to increase the sensitivity of detection of microorganisms. We found certain inhibitory substances in the propellant that prevented the maximum growth of our inoculum. These inhibitors had to be leached out to increase recovery efficiency. It was possible, for example, to not detect as many as 10^4 microorganisms per cm^3 in solid propellant if the leaching step was omitted. When the leaching step and certain additional steps were included, it was possible to detect as few as 10 microorganisms per cm^3.

Improvements were also made in the pulverization of the solid. Through the use of saws possessing certain specific characteristics, it was possible to pulverize solid propellant more effectively than any other previously used method. A thermistor was attached to the back one inch of blade and a slow rate of sawing was used. The rate of sawing was so regulated that an increase in temperature of not more than $1^{\circ}C$ resulted. Because the temperature at the saw tooth-propellant interface may be several degrees higher than that indicated by the thermistor, the rate of sawing was adjusted to minimize possible thermal inactivation of microorganisms. The procedures are summarized in Figure A-1.

Microscopically innumerable fine cracks and checks were noted on the sawed particles which were noted to have continuity with the interstices of even the largest particles. The larger particles appeared to result from

17°

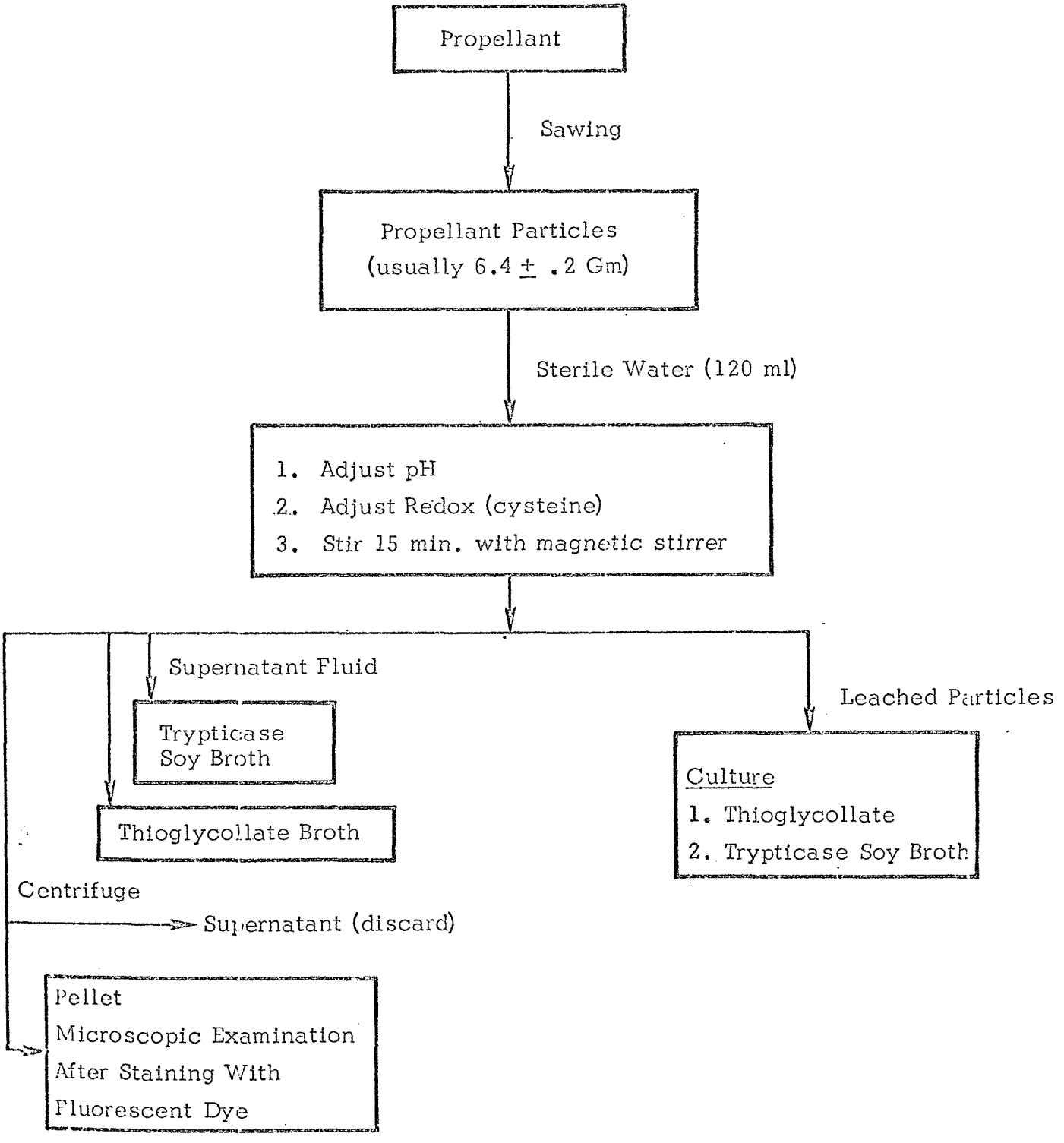

Figure A-1. Propellant Culturing Procedure

the fusion of smaller particles. This fusion effect was likely due to the sticky adherent surfaces of the solid propellant material. This fusion effect could not be reversed by treatment with the Waring blender, sonication, or by the treatment with wetting agents. Although Tween-80* was included in the culture media and did provide superior recoveries, it does not appear that such improved recoveries were the result of inhibition of particle agglomeration.

Leaching of Propellant

The propellant particles were extracted with sterile distilled water and stirred with a teflon coated magnetic stirring bar under sterile conditions. The optimum ratio of leaching fluid to solid propellant was established. The redox potential of the leached propellant and the supernatant fluid was measured and this potential was adjusted to that considered optimum for each microorganism tested. The supernatant fluid at the end of the leaching period was removed, the pH adjusted to 7.2 - 7.4 using sterile 0.1 N acid or base (except in the case of Ulocladium or other fungi the pH was adjusted to 4.0 - 4.5). Tween-80 was incorporated in the growth medium (Figure A-2).

A Reappraisal of Assay Techniques in Solid Materials

The application of steps previously noted in this addendum have increased the sensitivity of the detection of inoculated organisms from a level of 10^5 to 10^1 per cm^3. Recovery of microorganisms from inoculated solid propellant utilizing microtome sectioning technique was uniformly poor as noted previously in Phase III discussion.

Because of the large order magnitude of improvement in the recovery of small members of microorganisms from propellant, our studies were extended to solids from which previously large inocula of organisms (10^8/gram)

*polyoxyethylene-sorbitan-monoaleate (Atlas Powder Co.)

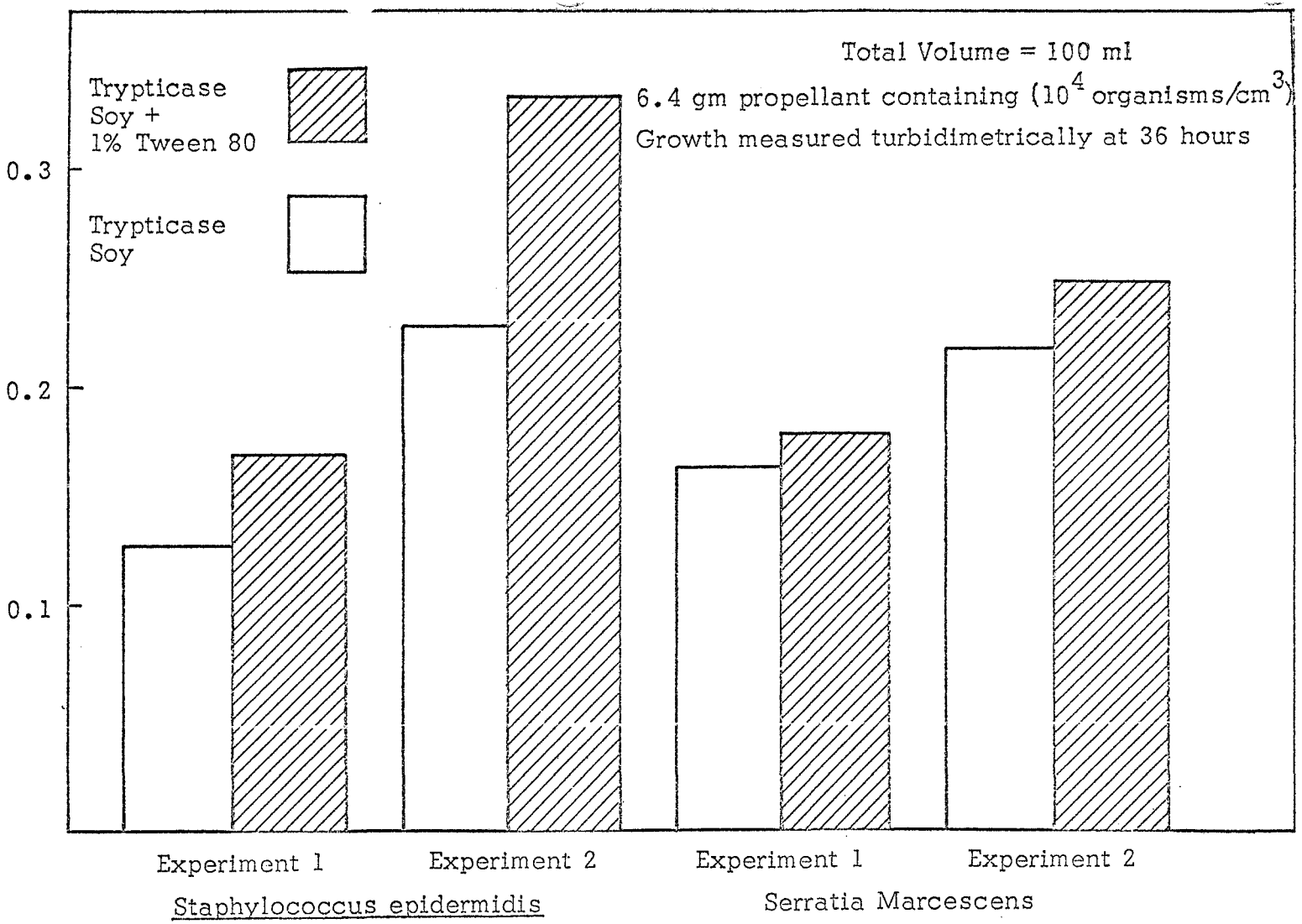

Figure A-2. Effect of Tween 80 on Growth of Selected Bacteria in Propellant as Determined Turbidimetrically

gave in many instances negative recoveries of microorganisms. By applying these improved methods, it was possible to detect as few as 100 microorganisms per cm^3 from two types of epoxy resins. It therefore is apparent that the culture techniques originally developed for solid propellants are applicable in the detection of microorganisms in solid plastic materials (Stycast 2850 GT, Eccocoat IC2) from which recoveries were previously unsatisfactory.

Recommendations

In extending the assay procedures which were devised and optimized for propellant recovery studies, it would be desirable to study in greater detail the pulverization of the various classes of solid materials. If the pulverization was so drastic as to cause the rupturing of the cell wall in the microorganism, it would be likely that the organism could be detected by cultural techniques. The process of freeing the microorganisms from encasement in rigid solids may tend to cause irreversible cellular damage. Although the particle size achieved by the pulverization may approach that of the microorganism, this process may be less desirable than one which produces larger particles.

In the latter case, communicating microcracks may exist which would provide both for diffusion of nutrients to and growth of the encased microorganisms from internal sites.

Because of the occurrence of toxic compounds in certain types of solid materials, it will be important to establish optimal methods for the leaching of these substances free from the pulverized solid. A large variety of plastic polymers are polymerized by catalysts which may inhibit the growth of microorganisms. These catalysts vary according to the type of plastic and each represents a separate problem. Unreacted monomers when present may also inhibit the growth of microorganisms. It is obvious that in addition to optimizing the pulverization and leaching procedures that it will be necessary to devise methods for neutralizing or inactivating both the soluble leachable inhibitors and the inhibitors present within the solids themselves.

Further investigation of culture media development will be required to optimize the isolation of microorganisms from solids. Since the type of biochemical injury or event which results in the devitalization of the microorganism may not be definable, it may be necessary to add a variety of substances which tend to counteract these events. In order to reactivate such inhibited organisms it may be necessary to add growth factors and metabolic constituents to the medium which may not normally be required by the organism under other conditions. The addition of an excess of the known required vitamins, amino acids, sugars and growth factors at levels which do not interfere with growth or metabolism of the organism is highly desirable. Adjustment of trace metal content, ionic strength, pH and redox potential may also be desirable.

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